JOURNAL OF THE NATIONAL CANCER INSTITUTE

November 1957
Volume 19 • Number 5

A Publication of the
U. S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Public Health Service
National Institutes of Health

JOURNAL OF THE NATIONAL CANCER INSTITUTE

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JOURNAL OF THE NATIONAL CANCER INSTITUTE

NATIONAL INSTITUTES OF HEALTH • Public Health Service

Volume 19
Number 5

November 1957

A Transplantable Acute Leukemia in an Inbred Line of Rats [1,2]

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Published reports from other laboratories (*1–3*) indicate that leukemia is a rare form of malignant disease in untreated rats. Transplantable leukemias have been reported by Shay *et al.* in random-bred rats (*4*). Lewis (*5*) reported a transplantable lymphoma in her inbred line of Wistar rats and recently Iglesias and Mardones (*6*) reported 2 transplantable lymphocytic-type leukemias in our A × C line 9935 rats.

No leukemia was observed (*7*) in a population study of malignant diseases in the colony of inbred rats maintained by the authors. A study of the incidence of malignant diseases (*8*) among 9,566 rats, 12 or more months of age, from the first 20 brother × sister generations of Fischer line 344 and Copenhagen line 2331 and their F_1 and backcrossed hybrids revealed no cases of spontaneous leukemia, though reticulum-cell sarcoma was found to be the most frequent type of neoplasm in Fisher line 344 rats and their hybrids. Since the population data have been analyzed, spontaneous leukemia has been observed in a small number of untreated rats of several strains in this colony and lymphatic leukemia was reported (*9, 10*) in diethylstilbestrol-treated and estrogen-treated rats of 4 inbred lines including Fischer line 344.

In the population studies, some cases of leukemia were undoubtedly missed in routine *post-mortem* examinations because moderately enlarged livers, spleens, and lymph nodes were not preserved for microscopic examination unless the rat received treatment with a potential carcinogenic or therapeutic agent, served as an untreated control for such a treated rat, or was the donor of tumor tissue for a subsequent transplantation. The presence of leukemia was missed in the gross *post-mortem* examination of the current case.

History

The acute leukemia to be described occurred spontaneously in a rat bearing the transplanted acetylaminofluorene-induced mammary adenocarcinoma IRC 741 (fig. 1), which was used to transfer that neoplasm

[1] Received for publication August 16, 1955.
[2] This study was supported by a gift from Dr. and Mrs. Julius Hyman.
[3] Established by a grant from the Damon Runyon Memorial Fund for Cancer Research.

from the 21st to the 22nd transfer generation. It was detected and preserved because it was accidentally transferred with the adenocarcinoma to the new hosts. Routine histological examination of the tissues, preserved at the time of the transfer, demonstrated the presence of the new neoplasm after a difference in the behavior of the recipient rats had been observed. Microscopic examination of a section of the tumor adjacent to the part used for transplantation revealed an adenocarcinoma like that observed in previous generations of IRC 741, except numerous malignant white cells were in the blood vessels of the tumor. In figure 2 a section of IRC 741/24A after 3 subsequent transfers shows massive contamination with the new neoplasm.

The primary host of the adenocarcinoma was a female rat, Fischer line 344, from the 47th brother × sister generation of the line. All the 235 rats inoculated with the tumor for the first 21 generations were of the same inbred line as the primary host. Each rat received subcutaneously by trocar 1 or 2 grafts weighing approximately 3 mg. As shown in table 1 the average interval between transfers was 54 days. All the grafts grew progressively and the rats had large ulcerated tumors when they died or were killed at an average of 61 days after inoculation. Sections of the tumors, axillary and mediastinal lymph nodes, and 4 sections of lungs from 118 of these rats, including all donors of tumor tissue used to propagate the adenocarcinoma, were preserved for microscopic examination. All the tumors proved to be adenocarcinomas similar to the one shown in figure 1. Lymph-node or lung metastases (fig. 3), or both, were identified in sections from 65 of these rats and, in addition, 2 rats had metastases in the kidneys. There was no evidence of leukemia in any of the rats.

Origin and Description of Primary Leukemia

The host of the primary leukemia was a Fischer line 344 female rat from the 51st brother × sister generation of the line. Three mg. grafts from 1 of the adenocarcinomas of the 20th transfer generation of IRC 741 were transplanted subcutaneously to both sides of this rat when it was 359 days old. Thirty-five days later each tumor measured 6.0 × 3.0 × 2.0 cm. and the rat was sacrificed to obtain grafts for the 22nd transfer of IRC 741. Subsequent microscopic examination of the tissues preserved at necropsy showed that the tumor was an adenocarcinoma and that there

TABLE 1.—*Transplantation history of IRC 741*

Tumor generation	Average no. days to transfer	No. rats inoculated	Transplantation history	Average no. days to death
1–21	54	235	++	61
22A	35	10	++	28
23A	34	10	+	26
24A	24	9	+	24
25A	20	10	+	28
26–53	19	422	+	20
53–80	15	444	+	18
80–105	15	447	+	17

were abnormal cells in the blood vessels of the tumor and in sections of the lungs and lymph nodes.

The rats of the 22nd transfer generation survived an average of only 28 days. One was sacrificed on the 34th day to obtain tissue for the next transfer. The average interval between transfers and the average survival of the inoculated rats shown in table 1 became progressively shorter. In the shortened interval between transfers, the adenocarcinoma did not compete successfully with the leukemia and was lost at the 26th transfer generation. No evidence of the adenocarcinoma has been observed in later transfer generations. The average survival period for the rats inoculated with generations 26 to 53 was 20 days. For the 2 succeeding years the average survival period has remained fairly stable—18 days for generations 53 to 80 and 17 days for generations 80 to 105.

Description of Transplanted Leukemia

The practice of transferring the disease by subcutaneous trocar implantation of a 2 to 4 mg. graft of tumor tissue taken from the subcutaneous growth at the site of the previous inoculation has been continued. The tumor is now in the 115th transfer generation. Like most isologously transplanted neoplasms, the sex and age of the host was found to have no influence on the rate of growth or the survival of the inoculated rats. In each generation a diffuse subcutaneous mass has been palpable about 10 days after inoculation. Death usually occurred between the 14th and 25th day. The *post-mortem* examination usually revealed a large edematous and hemorrhagic mass of tissue at the site of inoculation which had infiltrated both the skin and underlying muscle. Large hemorrhagic axillary and mediastinal lymph nodes, congested lungs, and enlarged livers and spleens were also commonly observed. Microscopic examination of these tissues revealed massive infiltration of the subcutaneous tissues and underlying muscle, right axillary and mediastinal lymph nodes, lungs, liver (fig. 5), and spleen with predominantly large round cells containing round to ovoid nuclei. Stained smears of the peripheral blood contained predominantly large, round to ovoid cells showing considerable variation in size and shape—frequently with pseudopodia-like processes. The nuclei were round to ovoid and contained finely stippled chromatin. The cytoplasm was usually basophilic and contained no definite granules. The living cells, however, on a warm stage with dark field, showed definite ameboid motion with fine dustlike particles streaming into the forward process as is characteristic of the cells in monocytic leukemia. Cells fixed with extended processes were frequently observed in the smears (figs. 4 and 6). Mitotic figures were also commonly observed in cells of the peripheral-blood smears.

Peripheral-Blood Picture

Examination of the peripheral blood up to the 12th day after inoculation frequently showed very little change from the normal picture. Table 2 shows that between the 12th and 19th day after inoculation

TABLE 2.—*Frequency distribution for the white-cell count in rats at specific intervals after inoculation with IRC 741*

White blood cells per mm.³	Days after inoculation:										
	0	1–6	7–12	13	14	15	16	17	18	19	20
400,000					1		1	2	1	1	1
300,000					1		1	18	3	4	2
200,000						1	4	24	12	5	4
100,000			1	2	1	5	7	17	9	9	8
90,000			1	1		1	2	1			
80,000							1	1		1	4
70,000					3	1	1	2			
60,000				2	1	1	3	1			
50,000			2	3	4	2				1	
40,000			1	2	6	4	1	2			1
30,000		3	14	6	3	4	1	1	1	1	1
20,000	4	8	23	4	14	4	3	3	2		1
10,000	36	47	81	3	9	8	7	4	3	1	3
0,000	14	21	23	1	5	5		1	3		
TOTAL	54	79	146	24	48	36	32	77	34	23	25
Thousands per mm.³											
Mean	13. 1	14. 10	19. 10	47. 5	48. 9	53. 1	114. 2	213. 1	176. 3	201. 7	153. 6
±P. E.	0. 50	0. 54	0. 88	5. 0	7. 6	6. 4	12. 7	8. 9	13. 6	15. 6	15. 3
S.D.	5. 50	7. 10	15. 80	36. 5	78. 1	56. 8	106. 8	115. 9	117. 4	110. 0	113. 3
±P. E.	0. 35	0. 38	0. 62	3. 6	5. 4	4. 5	9. 0	6. 3	9. 6	11. 0	10. 8

the white-cell count was elevated sometimes as high as 400,000 cells per mm^3. The high counts always showed a preponderance of abnormal cells. The frequency distribution and the mean white-cell count at stated intervals after inoculation is shown for 573 rats in table 2. The mean rose from 13.1 ± 0.50 thousand per $mm.^3$ at the time of inoculation to 213.1 ± 8.9 thousand on the 17th day after inoculation. After the 17th day some of the rats showed a reduced white-cell count of the peripheral blood. The low counts at this stage were practically 100 percent malignant cells and usually represented a reduction from a previously higher count. Some rats died with very high counts while others, terminally, showed a reduced count even when all the vessels and tissues appeared to be filled with abnormal white cells.

Typical white-cell counts are shown in table 3 for 3 rats from IRC 741/39A and for 3 rats from IRC 741/42A. Rat #3 in the first group had a count of only 8,700 on the 17th day that increased to 442,000 on the 22nd day when it was killed for transplantation. Rat #5 and #9 from the same group had counts of 305,000 and 235,000, respectively, on the 17th day. These were reduced to 48,250 and 15,625 on the 22nd day, when the former died with macroscopic and microscopic evidence of leukemia. The counts of the 3 rats in the 2nd group were all elevated on the 17th day. The 2 that were examined on the 19th day were further elevated to 493,000 and 366,000. They died on the 21st and 22nd day after inoculation. Figures 4 and 6 were made from a smear of the peripheral blood of a rat on the 17th day after inoculation with IRC 741/39A when the count was 330,000.

TABLE 3.—*White-cell count for rats inoculated with IRC 741*

Rat No.	White blood cells before inoculation	Tumor generation	White blood cells after inoculation			Days to death
			10 days	17 days	22 days	
3	15, 200	741/39A	17, 200	8, 700	442, 500	*
5	7, 700	"	11, 700	305, 000	48, 250	22
9	13, 200	"	12, 900	235, 000	15, 625	28
			14 days	17 days	19 days	
3		741/42A	19, 300	353, 000	—	*
8		"	24, 000	193, 000	493, 000	21
9		"	17, 600	216, 000	366, 300	22

*Killed for tumor transplantation.

Transmission by Blood and Liver

It was found that the leukemia was transmitted readily by subcutaneous trocar implantation of fragments of liver or subcutaneous injection of whole blood, or by intraperitoneal injection of a 1:5 or a 1:50 dilution of whole blood. Regardless of the method of transfer, the course and appearance of the disease was similar, but rats receiving intraperitoneal injections did not develop subcutaneous tumor masses and they usually

TABLE 4.—*Results of experiments with inoculum from several tissues*

Tumor/ generation	Material used for injection	Route of administration	No. of rats inoculated	Average days to death
741/33A	Subcutaneous tumor	Subcutaneous	10	18
	Liver	Subcutaneous	10	14
741/41A	Subcutaneous tumor	Subcutaneous	9	21
	Whole blood—0.2 cc	Subcutaneous	1	12
	Whole blood—0.2 cc	Intraperitoneal	10	10
741/41B	1:50 Dilution of whole blood—0.2 cc	Subcutaneous	2	18
		Intraperitoneal	5	15
741/43A	Subcutaneous tumor	Subcutaneous	8	19
	1:50 Dilution of whole blood—0.2 cc	Intraperitoneal	3	11

had ascites, fluid in the chest, more generalized involvement of the omentum and mesentery, and shorter average survival period than those receiving subcutaneous grafts. The results of some of these experiments are shown in table 4.

Susceptibility of Foreign Strains

In an attempt to survey the relative susceptibility of other inbred strains in our colony to this neoplastic disease, 1 each of 12 different strains of rats were implanted subcutaneously with a graft from the subcutaneous growth of IRC 741/37A. Six of these rats (August line 990; A × C line 9935 Irish; A × C line 9935 piebald; Selfed line 36670; Zimmerman line 61; and Copenhagen line 2331) died with leukemia in an average of 36 days. The negative strains were Yoshida line 38366, Avon line 34986, August line 28807, August line 35322, Marshall line 520, and Wistar. In a later transfer (IRC 741/46A) 10 rats, *i.e.*, 2 each of the 5 strains that had appeared from the previous test to be susceptible, were implanted subcutaneously and 9 died with leukemia in an average of 44 days. The 1 negative was an A × C line 9935 Irish rat. In some of these rats the only evidence of the disease was manifested by the growth of the tumor mass at the site of inoculation. One such rat, a Zimmerman line 61 male, was used as a source of inoculum to transfer the disease to Fischer line 344 rats. For 24 generations, the subsequent course of the disease in the Fischer line 344 rats paralleled the course of the disease in the series of rats that had been continuously implanted from isologous hosts. One passage through a host of a foreign strain failed to alter the course of the disease. In a later transfer (IRC 741/97B) 10 rats of the Zimmerman line 61 were inoculated and 9 died with leukemia in an average of 39 days. In addition, 2 rats each of 4 negative strains (Yoshida line 38366, August line 28807, August line 35322, and Selfed line 36670) were injected intraperitoneally with 0.2 cc. of a 1:5 dilution of whole blood from a leukemic Fischer line 344 rat of the 42nd transfer generation.

One of the Yoshida rats died with leukemia after 26 days. Eight weanling rats of the August line 28807 and 3 weanling rats of the Marshall line 520 were inoculated subcutaneously with IRC 741/108A. Seven of the August rats and all 3 of the Marshall rats died with leukemia in an average of 23 days after inoculation. The weanling rats were apparently more susceptible than the adult rats of the same strain. Two mice each, of 5 strains (PL, AKR, C3H, C3Hf/He, and C57BL), were inoculated with IRC 741/37A but none showed any evidence of the disease.

Attempts to transfer the disease to adult and newborn isologous hosts by filtrates of diluted blood and other tissues and the response of the disease to some alkylating agents will be described in subsequent reports.

Summary

1) A spontaneous acute leukemia was transplanted as a contaminant in Fischer line 344 rats bearing transplanted mammary adenocarcinoma IRC 741.

2) The leukemia was found to be transplantable by subcutaneous implantation of 3 mg. grafts of tissue from the site of the previous inoculation, liver fragments, or subcutaneous or intraperitoneal injection of 0.2 cc. of whole blood or blood diluted 1:5 or 1:50 with saline.

3) The transplanted leukemia developed in 14 to 30 days in 100 percent of the inoculated adult rats of the same inbred line as the host of the primary neoplasm and in some rats of 9 foreign strains.

4) Palpable masses developed at the site of inoculation within 10 days. White-cell counts of the peripheral blood averaged more than 100,000 cells per mm.^{3} from the 16th day after inoculation.

5) Death resulted in an average of 17 to 20 days after inoculation and *post-mortem* examination revealed a diffuse infiltrative growth at the site of subcutaneous inoculation, large hemorrhagic adjacent axillary and mediastinal lymph nodes, congested lungs, and enlarged livers and spleens. Microscopic examination of these tissues showed massive infiltration of all the tissues with abnormal leukocytes.

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PLATE 77

FIGURE 1.—Transplanted adenocarcinoma IRC 741/11B, 66 days after subcutaneous inoculation. × 180

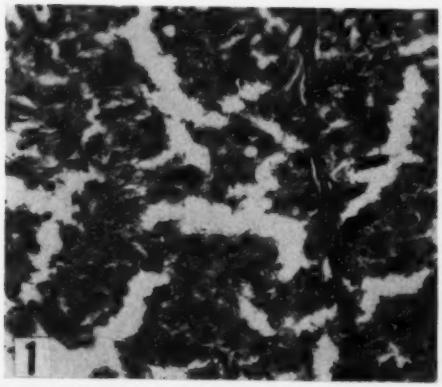
FIGURE 2.—Transplanted adenocarcinoma IRC 741/24A, 20 days after subcutaneous inoculation, showing contamination with leukemic cells. × 180

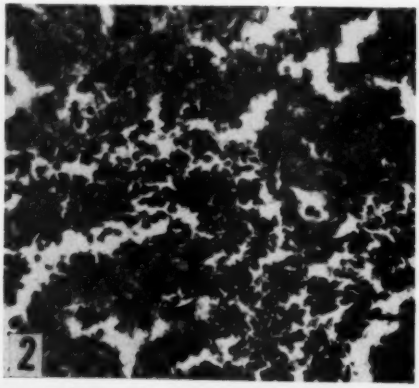
FIGURE 3.—Lung metastasis of IRC 741/11B, 66 days after subcutaneous inoculation. × 180

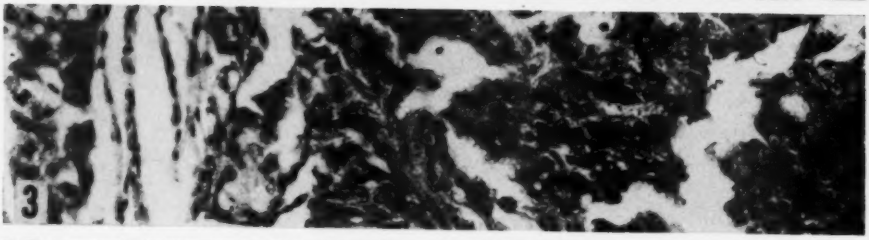
FIGURE 4.—Smear from the peripheral blood of a rat, 17 days after subcutaneous inoculation with IRC 741/39A. The total white-cell count was 330,000 cells per $mm.^3$ × 300

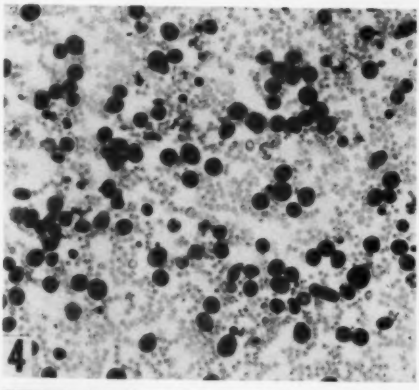
FIGURE 5.—Section from the liver of a rat, 17 days after subcutaneous inoculation of IRC 741/83A, showing blood vessel filled with leukemic cells and massive infiltration of substance of liver. × 180

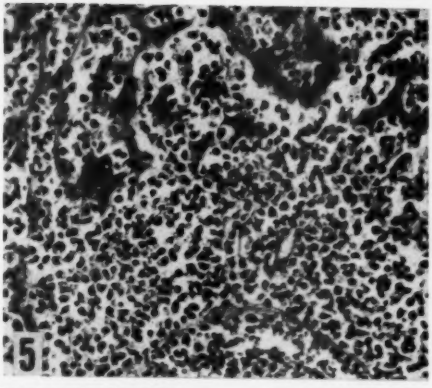
FIGURE 6.—Smear from the peripheral blood of a rat, 17 days after subcutaneous inoculation with IRC 741/39A. × 1100

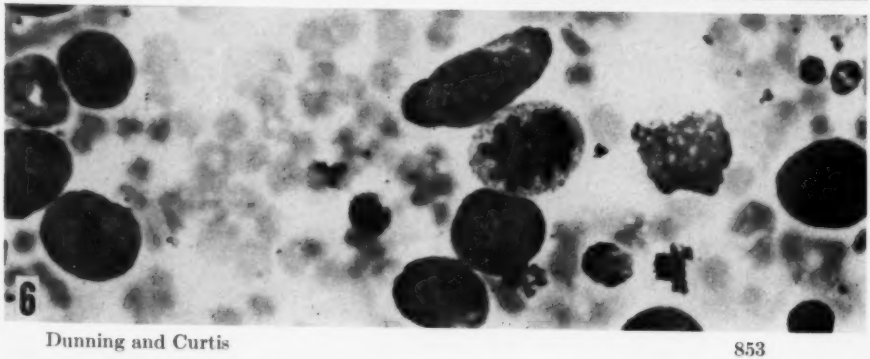












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Some Observations on C57BL Mice with Mammary Adenocarcinoma E0771 Receiving Combination Chemotherapy [1,2]

LOUIS BARVICK *and* LOUIS H. GOODSON,[3] *Chemistry Division, Midwest Research Institute, Kansas City, Missouri*

In a previous report (*1*) the use of combination chemotherapy against sarcoma 180 in BALB/c mice was based on the concept of biological antagonisms (*2–4*). The compounds employed at that time included amethopterin (AM) (*5–9*); *N,N*-bis(2-chloroethyl)aniline [aromatic nitrogen mustard (ANM)] (*10–13*); triethylenemelamine (TEM) (*14, 15*); and erythromycin (ER) (*16–18*). These compounds have been classified in the literature as antimetabolic, alkylating, and antibiotic agents.

In these experiments it was shown that combinations of specific doses of these drugs were more effective inhibitors of sarcoma 180 than the same doses of each drug employed individually. The question arose whether other strains of tumors in different strains of animals respond to treatment with these antagonists in a similar way. In order to obtain a partial answer, the original experiments were repeated, except for minor variations, substituting adenocarcinoma E0771 in C57BL mice for the sarcoma 180. As before, special emphasis was placed upon the effect of the drugs on the host, the tumor size, the food intake, body-weight loss, and histological changes of the tumor tissues.

Materials and Methods

The mammary adenocarcinoma E0771 was carried subcutaneously in the axillary region of C57BL mice. The implantations were made by trocar; the average implant was approximately 2 mm.^{3}. Treatments were begun on the 6th day; at this time all of the tumors were palpable and daily measurements by calipers were started. The body weights and food intake were recorded daily. The diet consisted wholly of Purina laboratory chow. Water was given *ad libitum* to all of the animals.

The 2 intermittent starvation experiments were begun on the 6th and 9th days of tumor growth, respectively. The first period of complete food removal was 4 days and was followed by a 2-day respite; then a

[1] Received for publication January 10, 1957.

[2] This investigation was supported in part by research grant C-802 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and in part by the Speas Foundation and the Midwest Research Institute.

[3] The authors wish to thank Dr. H. K. B. Allebach for the histologic examination of the slides and Dr. Raymond G. Stone, Jean Seay, and Martha Ibach for their technical assistance.

3-day starvation period followed by a 2-day respite. A 3rd period of starvation was begun but quickly terminated because of the poor condition of the animals.

The compounds used were AM, ANM, and ER.[4] These were suspended or dissolved separately in 3 percent gelatin solution prior to their injection by the intraperitoneal route. When combinations of 2 or 3 compounds were injected into a single animal, the compounds were injected in succession. Maximum-tolerated doses were used (table 1) and the schedule of 1 dose per day for 4 days per week was selected as determined previously.

Gross and microscopic studies of the tumor tissues were made. However, because the cellular changes observed were nonspecific, detailed discussion and photomicrographs have been omitted from this paper.

Results and Discussion

The experimental data are summarized in table 1 and the changes occurring in the animals and tumors during the treatment are shown graphically in the text-figures 1 through 5.

Experiments in which AM, ANM, and ER were employed singly to mice bearing E0771 showed that only AM at 3 mg. per kg. produced significant inhibition of tumor growth and prolongation of survival; these were 84 and 78 percent, respectively. The mouse plus tumor-weight gain in the AM-treated group was slight compared to the increases shown by the other treatments (text-figs. 1 and 2).

The 2-compound combinations (text-figs. 3 and 4) showed a general retarding effect on tumor growth but only AM+ANM, at 3+10 mg. per kg., gave a significant increase in survival time. On the 19th day, retardation was approximately 80 percent of that of the controls with an accompanying increase in survival time of 75 percent. Despite the above, AM alone was as effective as the combination, indicating that ANM contributed little to the result.

The 3-compound combinations of AM+ANM+ER (text-fig. 5) produced 80 to 93 percent retardation, but the survival time was not significantly increased.

The non-tumor-bearing mice (text-fig. 6) show the definite toxic effect of the combination treatments. It will be noted that the combinations of drugs and specific dosages administered to the non-tumor-bearing animals are the same as those administered to the tumor-bearing animals. A possible explanation of the high mortality in the non-tumor-bearing group lies in the fact that older animals were used for these experiments. Consequently, this experiment shows that the dosages employed in these studies are in excess of the maximum-tolerated doses.

It is interesting to note that the total starved mice bearing E0771 (text-fig. 7) showed approximately 70 percent tumor retardation in the 12-day growth period. For the same period AM-treated mice showed 80 percent retardation. Both starvation and treatment produced similar

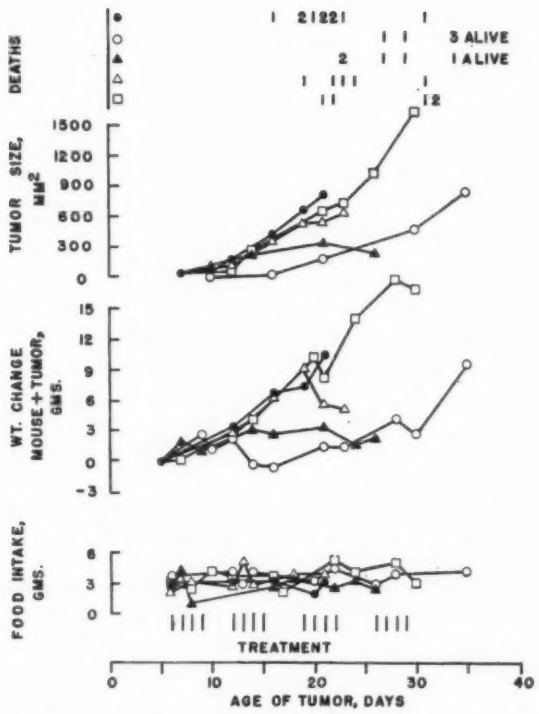
[4] We wish to express our thanks to Eli Lilly and Company for the sample of erythromycin and to Lederle Laboratories Division, American Cyanamid Company, for the amethopterin.

TABLE 1.—*Summary of combination therapy experiments on C57BL mice bearing mammary adenocarcinoma E0771*

Experiment*	No. mice	Daily dose (*mg./kg.*)	Tumor area (*mm.²*)				Mouse + tumor wt. change (*percent*)			Survival time (*days*)			
			No. doses	10th day	12th day	19th day	10th day	12th day	19th day	Average	Range	Percent increase	Text-fig. no.
1a. Control†	10		11	97	184	669	8	15	31	21.4	16–31	—	
AM	5	3.0	16	6	35	129	5	9	3	38.0	27–47	78	
ANM	5	20.0	13	78	163	310	6	12	18	29.0	23–44	36	1
ANM	5	10.0	12	53	120	535	7	13	44	23.8	19–31	11	
ER	5	25.0	16	47	111	513	5	13	39	27.6	21–32	29	
1b. Control	6		11	63	190	927	1	8	31	23.5	19–25	—	
AM	6	1.5	11	44	174	812	5	8	33	25.5	21–28	9	2
ANM	6	5.0	11	105	315	979	3	12	40	22.5	18–29	0	
ER	6	12.5	11	53	237	655	−2	2	21	21.7	19–25	0	
2a. Control†	10			97	184	669	8	15	31	21.4	16–31	—	
AM+ANM	10	3+20	16	17	35	115	−2	−2	−2	26.7	24–35	25	3
AM+ANM	10	3+10	16	7	16	112	1	1	1	37.4	24–53	75	
2b. Control	10		12	167	190	595	4	3	20	20.9	14–34		
AM+ER	10	3+25	12	10	24	150	−4	−6	6	29.3	12–42	40	4
ANM+ER	10	20+25	11	75	128	217	−3	−8	0	19.8	10–35	Less	
ANM+ER	10	10+25	12	99	159	466	−4	0	23	20.3	14–26	Less	
3. Control†	10			97	184	669	8	15	31	21.4	16–31	—	
AM+ANM+ER	10	3+20+25	12	5	11	111	−17	−18	2	21.7	10–48	1	5
AM+ANM+ER	10	3+10+25	12	1	7	47	−11	−11	−12	23.9	12–52	12	
AM+ANM+ER	6	1.5+5+12.5	12	23	69	327	0	−3	13	27.7	25–29	18	
4. Non-tumor-bearing animals													
AM+ER	5	3+25	5				−10	−12	—	14.4	12–18	—	
ANM+ER	6	10+25	12				−7	−3	−6	—	‡	—	6
AM+ANM+ER	5	3+20+25	7				−17	−10	—	12.8	9–22	—	
AM+ANM+ER	6	1.5+5+12.5	12				0	−2	1	—	‡	—	
5. Intermittent feeding	10			43	115	328	−19	4	7	24.8	10–37	16	
Total starvation	5			28	56	0	−22	−36	—	13.0	12–14	0	
Total starvation	5			48	106	0	—	−24	—	15.6	14–19	0	7

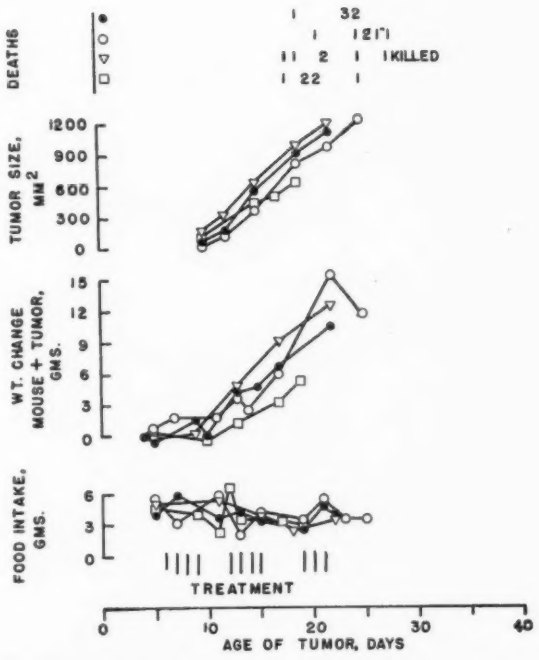
*AM=amethopterin; ANM=*N,N*-bis(2-chloroethyl)aniline; ER=erythromycin. †Experiments 1a, 2a, and 3 were run simultaneously and employed the same control.

‡Most animals survived, *see* text-figure 6.



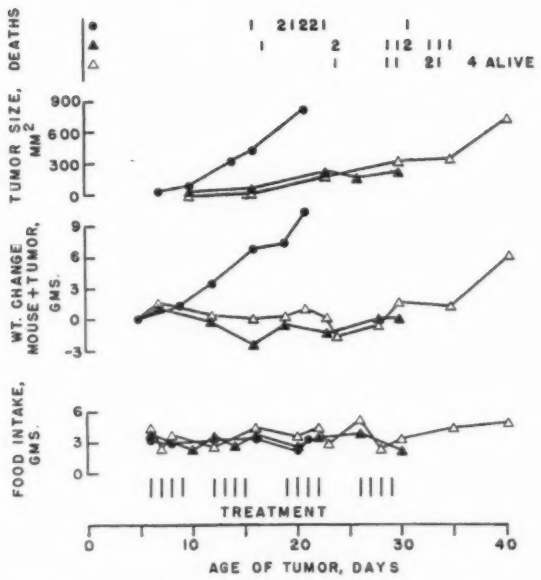
TEXT-FIGURE 1.—Comparison of tumor growth, mouse plus tumor weight, food intake, and days on which death occurred for mice bearing mammary adenocarcinoma E0771 and receiving injections of:

○—3 mg. per kg. of amethopterin
▲—20 mg. per kg. of *N,N*-bis(2-chloroethyl)aniline
△—10 mg. per kg. of *N,N*-bis(2-chloroethyl)aniline
□—25 mg. per kg. of erythromycin
●—Controls, 3 percent gelatin solution



TEXT-FIGURE 2.—Comparison of tumor growth, mouse plus tumor weight, food intake, and days on which deaths occurred for mice bearing mammary adenocarcinoma E0771 and receiving injections of:

○—1.5 mg. per kg. of amethopterin
▽—5.0 mg. per kg. of *N,N*-bis(2-chloroethyl)aniline
□—12.5 mg. per kg. of erythromycin
●—Controls, 3 percent gelatin solution

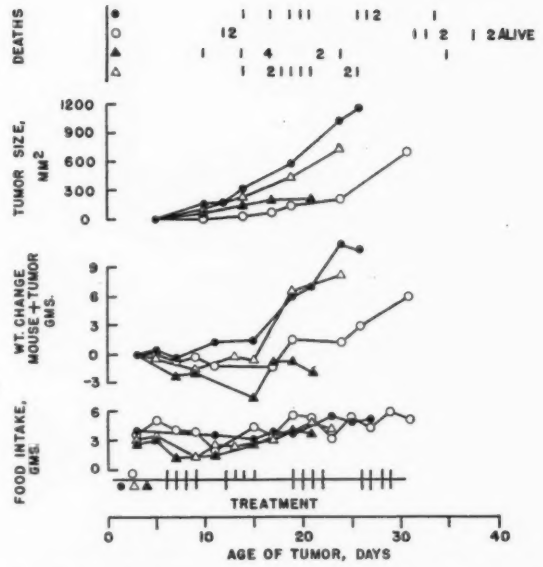


TEXT-FIGURE 3.—Comparison of tumor growth, mouse plus tumor weight, food intake, and days on which deaths occurred for mice bearing mammary adenocarcinoma E0771 and receiving injections of:

▲—Combination of 3.0 mg. per kg. of amethopterin and 20 mg. per kg. of *N,N*-bis(2-chloroethyl)aniline

△—Combination of 3.0 mg. per kg. of amethopterin and 10.0 mg. per kg. of *N,N*-bis(2-chloroethyl)aniline

●—Controls, 3 percent gelatin solution



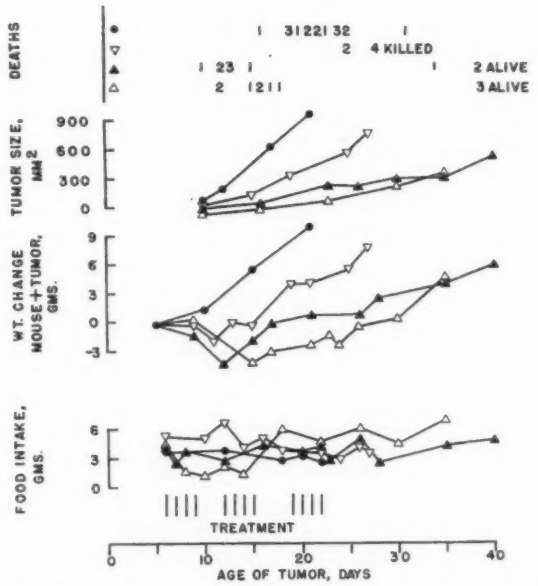
TEXT-FIGURE 4.—Comparison of tumor growth, mouse plus tumor weight, food intake, and days on which deaths occurred for mice bearing mammary adenocarcinoma E0771 and receiving injections of:

○—Combination of 3.0 mg. per kg. of amethopterin and 25 mg. per kg. of erythromycin

▲—Combination of 20 mg. per kg. of *N,N*-bis(2-chloroethyl)aniline and 25 mg. per kg. of erythromycin

△—Combination of 10 mg. per kg. of *N,N*-bis(2-chloroethyl)aniline and 25 mg. per kg. of erythromycin

●—Controls, 3 percent gelatin solution



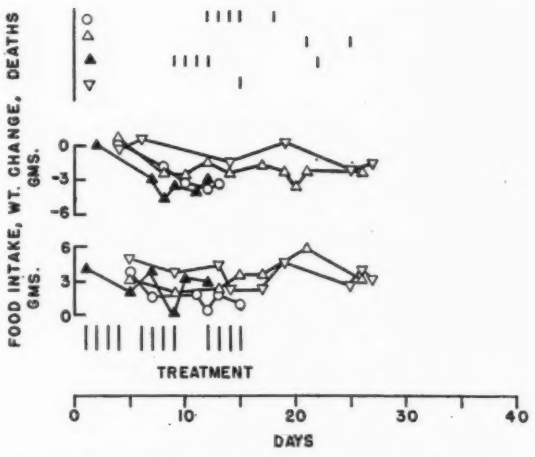
TEXT-FIGURE 5.—Comparison of tumor growth, mouse plus tumor weight, food intake, and days on which deaths occurred for mice bearing mammary adenocarcinoma E0771 and receiving injections of:

▽—Combination of 1.5 mg. per kg. of amethopterin plus 5 mg. per kg. of *N,N*-bis(2-chloroethyl)aniline plus 12.5 mg. per kg. of erythromycin

△—Combination of 3 mg. per kg. of amethopterin plus 20 mg. per kg. of *N,N*-bis(2-chloroethyl)aniline plus 25 mg. per kg. of erythromycin

▲—Combination of 3 mg. per kg. of amethopterin plus 10 mg. per kg. of *N,N*-bis(2-chloroethyl)aniline plus 25 mg. per kg. of erythromycin

●—Controls, 3 percent gelatin solution



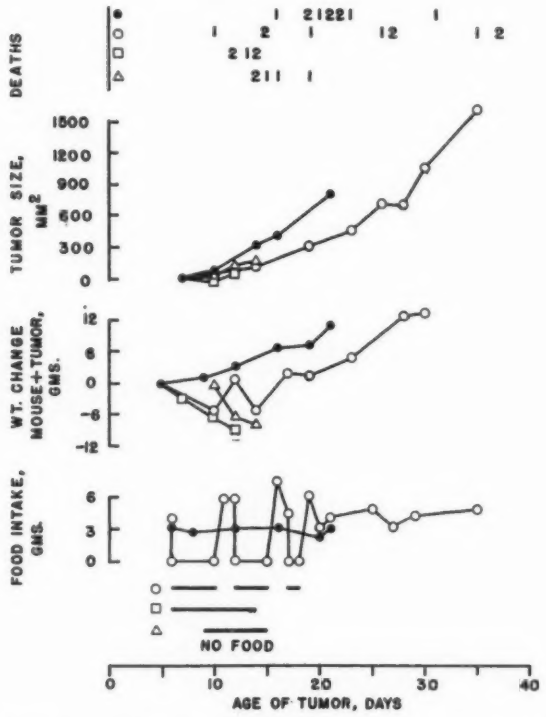
TEXT-FIGURE 6.—Effects on body weight, food intake, and survival of non-tumor-bearing mice receiving injections of:

○—Combination of 3 mg. per kg. of amethopterin and 25 mg. per kg. of erythromycin

△—Combination of 10 mg. per kg. of *N,N*-bis(2-chloroethyl)aniline and 25 mg. per kg. of erythromycin

▲—Combination of 3 mg. per kg. of amethopterin plus 20 mg. per kg. of *N,N*-bis(2-chloroethyl)aniline plus 25 mg. per kg. of erythromycin

▽—Combination of 1.5 mg. per kg. of amethopterin plus 5 mg. per kg. of *N,N*-bis(2-chloroethyl)aniline plus 12.5 mg. per kg. of erythromycin



TEXT-FIGURE 7.—Comparison of intermittent and total starvation on tumor growth, mouse tumor weight, and survival of mice bearing mammary adenocarcinoma E0771:

○—Intermittent feeding
□—Total starvation begun on the 6th day after tumor implantation
△—Total starvation begun on the 9th day after tumor implantation
●—Controls, fed *ad libitum*

effects. The intermittently fed mice also paralleled the treated tumor-bearing animals in the early stages of growth, and on the 19th day, retardation was approximately 45 percent of the control average. In table 1, it is seen that weight loss at 10 days' growth was quite excessive in both groups.

The results obtained here are similar to those found for sarcoma 180 in BALB/c mice (*1*), although the tumors and mice were of different species.

Conclusion

None of the various combinations of amethopterin (AM), *N,N*-bis(2-chloroethyl)aniline [aromatic nitrogen mustard] (ANM), and erythromycin (ER) administered to C57BL mice bearing mammary adenocarcinoma E0771 inhibited tumor growth or increased the survival over that produced by AM administered alone at the 3 mg. level.

In the 2 compound combinations, AM plus ANM was equal to AM alone in retarding tumor growth and lengthening survival time.

The 3 compound combinations retarded the tumors at the expense of host survivability.

The E0771 adenocarcinoma was retarded by AM and ANM, as was sarcoma 180 previously (*1*), indicating a similarity of response by the different host and different tumor.

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Mammalian Chromosomes *In Vitro*. VIII. Heteroploid Transformation in the Human Cell Strain Mayes [1,2]

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One of the recent advances in tissue culture is the establishment of numerous cell strains, which furnish useful material for various branches of biological and medical research because of their rapid growth and ease of maintenance in a variety of culture media. These cell strains originated from both malignant and nonmalignant tissues. Strains were derived from biopsies of solid neoplasms (*e.g.*, HeLa, KB), from solid normal tissues (*e.g.*, Ch-L, FF), peripheral blood (the Oregon J strains), suspended cells in fluids (Maben, McCoy), or even from single cells (NCTC clone 929 of strain L, S-3).

One of the important discoveries in this field was the capacity of the strains of nonmalignant origin to produce tumors. Earle and his associates were able to grow sarcomas in mice by inoculating cells of strain L (*1, 2*) and its clonal derivation, strain 929 (*3*). Moore, Southam, and their collaborators (*4, 5*) performed homotransplantation of various human cell strains into volunteer patients to detect whether or not the cells, after thriving in tissue-culture media for a certain period of time, would be able to produce tumors *in vivo*. The striking finding was that not only the originally malignant cell lines, such as HeLa and H.Ep.s, but also a number of the so-called "normal" strains produced tumors. These results suggest that the so-called "normal" strains, although established from nonmalignant tissues, have changed their nature during the period of cultivation *in vitro*. In other words, there seems to be a mechanism of carcinogenesis present in the culture methods for strain establishment.

The results obtained by Moore and her associates have been checked by a number of investigators on experimental animals, principally with the methods developed by Toolan (*6, 7*) for heterologous transplantation. Several authors (*8*) found positive results in producing tumors with human cell strains.

[1] Received for publication May 10, 1957.

[2] Supported in part by grants CP-12J and CP-84 from the American Cancer Society, Inc., and DRG-269B from the Damon Runyon Memorial Fund for Cancer Research.

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[6] The authors wish to express their appreciation to Mrs. Mildred Finerty and Mrs. Ursula Hild for their technical assistance.

It has long been known that neoplasms usually possess abnormal chromosome numbers. An interesting feature in the cytology of cell lines (*9, 10*) is that all the fast-growing strains examined so far are heteroploid. The modal chromosome number may vary from strain to strain or, possibly, from generation to generation; none of the strains possesses a true diploid- or tetraploid-chromosome constitution. Because normal tissues generally exhibit extensive diploidy *in situ* and in primary cultures, the change of ploidy in the cell strains apparently occurs after repeated subculturing while obtaining the cell line.

To prove that the ploidy does change during tissue cultivation, data is reported here on 1 human cell strain that was followed cytologically until a transformation to heteroploidy was reached. The relationship between the chromosomal transformation and the malignant conversion remains to be investigated.

Material and Methods

The material for this study was obtained on January 25, 1956, from the synovial lining of the left leg of a John Sealy Hospital patient (male, No. 2549-B). The patient had no malignant diseases. The tissue was cut into fragments and grown in T flasks with a fluid medium consisting of 50 percent Gey's solution, 45 percent human ascitic fluid, and 5 percent chick embryonic extract. The fluid was routinely changed twice a week. From subculture #2 to subculture #9, sample cells were taken from each generation and set up for roller-tube cultures in regular chick plasma clots.

After a certain period of growth, the cells on cover slips in roller tubes were treated with a hypotonic solution, fixed with methyl alcohol, and stained with May-Grünwald-Giemsa. These preparations provided the material for this study.

Strain Mayes grew profusely in various culture media until an unfortunate yeast contamination occurred in November, 1956, and then was lost.

Results

Subculture #2: (Age: approximately 3 months), 2 slides.—Cultures set up on April 30, 1956, and fixed on May 15, 1956. All cells were spindle-shaped. Only 1 metaphase figure was available for the record. This had about 47 chromosomes.

Subculture #3: (Age: nearly 4 months), 5 slides.—Cultures set up on May 15, 1956, and fixed on May 31, 1956. All cells were spindle-shaped and no mitotic figure was found.

Subculture #5: (Age: 4½ months), 12 slides.—Cultures set up on June 8, 1956, and fixed on June 13, 1956. All cells were spindle-shaped (fig. 1). A number of mitotic figures were seen and most of them were countable. Out of 49 cells showing diploid-chromosome constitution, 31 gave unequivocal counts of 46 (fig. 3). At least 4 cells clearly exhibited 47 chromosomes. Among the 6 cells showing subdiploid counts, 5 contained 45 chromosomes and 1 contained 44. However, none was clear enough to

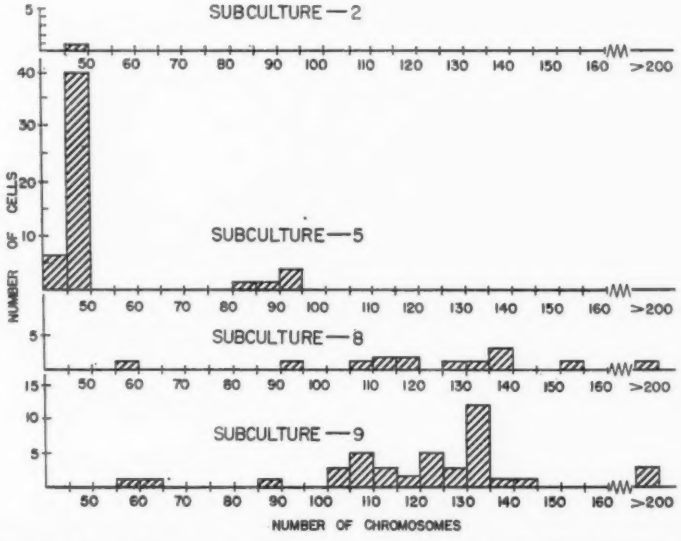
warrant a definite statement. The counts helped to prove that the stem-line number for normal human cells is 46 (*11*, *12*), as far as this individual is concerned. In the outgrowth zone of 1 explant, several apparent polyploid cells were found. Unfortunately, the chromosomes of these cells were fuzzy in outline and exact counting was not possible. However, rough counts on 6 of the polyploids (1 cell from another preparation) revealed that 4 of them were true tetraploids. Text-figure 1 summarizes the data in a graphic form.

Subculture #6: (Age: 5 months), 5 slides.—Cultures set up on June 22, 1956, and fixed on July 5, 1956. Most of the cells were spindle-shaped. No mitotic figures were found.

Subculture #7: (Age: 6½ months), 9 slides.—Cultures set up on August 6, 1956, and fixed on August 25, 1956. Most of the cells were spindle-shaped, but a few cultures contained many, somewhat flattened, cells. No mitotic figures were found.

Subculture #8: (Age: 6⅞ months), 10 slides.—Cultures set up on August 14, 1956, and fixed on August 25, 1956. Most of the cells assumed a flattened outline but a few remained spindle-shaped. Mitotic figures were numerous but a great proportion showed fuzzy chromosomes and counts were not possible. Fifteen cells were counted. The lowest chromosome number registered was 60, and 13 of the 15 cells had more than 100 chromosomes per cell (text-fig. 1). From the uncountable cells, it was also clear that none of the cells were in the diploid range.

Subculture #9: (Age: 7 months), 5 slides.—Cultures set up on August 27, 1956, and fixed on September 6, 1956. Practically all the cells were


SUBCULTURE — 2
SUBCULTURE — 5
SUBCULTURE — 8
SUBCULTURE — 9
NUMBER OF CELLS
50 60 70 80 90 100 110 120 130 140 150 160 >200
NUMBER OF CHROMOSOMES

TEXT-FIGURE 1.—Distribution of chromosome numbers in 4 subcultures in strain Mayes.

the flattened type (fig. 2). Mitotic figures were abundant and many showed clear-cut chromosomes. Out of 43 cells counted, only 3 had chromosome numbers below the tetraploid mark. The stemline number seemed to be about 133 (text-fig. 1). Figure 4 represents a cell from this set of preparations.

Discussion

It is obvious from the data presented that the cells of strain Mayes did change their chromosomal pattern from diploidy to heteroploidy in the course of *in vitro* cultivation. It has been speculated (*10*) that the transformation of chromosomal pattern may be a phenomenon of adaptation and selection within a cellular population. Generation 5 was composed mainly of diploids with a few tetraploids and very few doubtful cases of heteroploids. Cells of generation 8 had practically all heteroploids of high order. It was unfortunate that generations 6 and 7 did not contain mitotic figures, which would aid in determining whether the transformation occurred by a gradual increase of heteroploids or by a sudden, total replacement.

Strain Mayes was unique in that most of the cells were secondary heteroploids with a stemline number in the 130's, whereas most of the heteroploid strains have stemline numbers between 65 and 85. Counts on generations 8 and 9 of strain Mayes seemed to indicate that the populations were still in a stage of adjustment, even though both were already secondary heteroploids. However, the samples were too small to warrant such a conclusion. Further, as Levan (*13*) has stated, the population may adjust its chromosome number constantly and 1 sampling count can never characterize a strain.

Our results with strain Mayes, in which there is a distinct coincidence of morphological transformation, polyploidization, and increase in growth rate, reveal what may apply to the derivation of other normal tissue strains. Of course, the number of generations required to attain a radical degree of polyploidy may differ markedly.

The work of Goldblatt and Cameron (*14*), with intermittent, repeated exposures of cultures of rat fibroblasts to an atmosphere of pure nitrogen in order to obtain malignant conversions, seems to suggest that the usual method of bottle culture may produce a modified atmospheric condition (perhaps a partial anaerobiosis) that might stimulate the transformation. If the malignant conversion and chromosomal changes are truly related, it also would mean that the metabolism of the heteroploid cells is more adapted to an anaerobiotic condition than that of the diploids. However, all these arguments are more or less speculative, and much more information is needed about the relationship between the malignant changes and chromosomal transformation.

Summary

Cells from the synovial lining of a human knee joint (nonmalignant) gave rise to a strain known as Mayes. Subculture #5 consisted mainly of diploid cells with 46 chromosomes. Samples of subcultures #6 and #7

showed no mitotic figures. However, practically all the cells in subcultures #8 and #9 were secondary heteroploids.

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PLATE 78

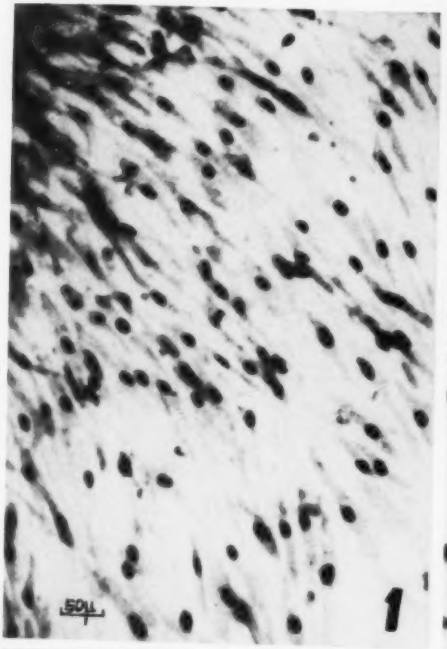
Photomicrographs showing cultural characteristics and chromosomes of strain Mayes. Methyl alcohol and May-Grünwald-Giemsa, all pretreated with hypotonic solution. Magnification for figures 1 and 2, *see* scale on figure 1; magnification for figures 3 and 4, *see* scale on figure 4.

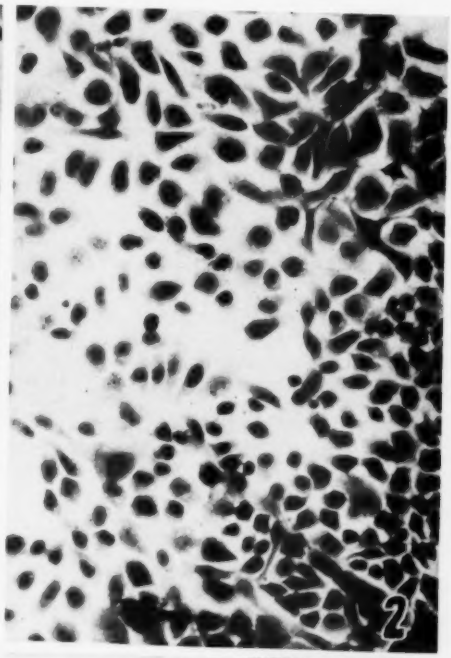
FIGURE 1.—Subculture #5. *Note* spindle-shaped cells.

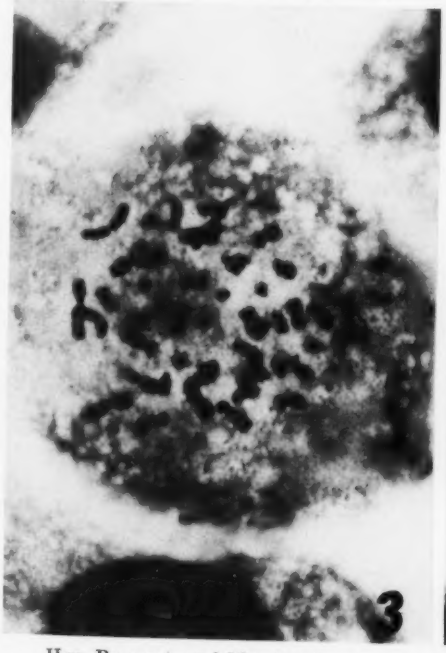
FIGURE 2.—Subculture #9. *Note* flattened cells.

FIGURE 3.—Cell from subculture #5 showing 46 chromosomes.

FIGURE 4.—Secondary heteroploid cell from subculture #9.







Hsu, Pomerat, and Moorhead

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Repopulation of Hematopoietic Tissues and Blood in Lethally X-Irradiated Mice by Homologous Bone-Marrow Cells [1, 2]

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Mice or rats are protected from the acute lethal effects of X irradiation of the whole body when they are injected with normal bone marrow (*1, 2*). Histological studies show that the hematopoietic tissues recover in injected animals only (*3–5*). The question arose as to whether the regenerated cells are derived from the irradiated recipients or from the injected bone marrow. The finding that homologous and heterologous bone-marrow cells also protect (*4*) suggests that the identity of the repopulating cells could be established if the heterologous or homologous cells could be distinguished from those of the irradiated hosts.

While the present investigation, based on the identification of homologous cells, was in progress, several other studies were reported in the literature based on the identification of homologous or heterologous cells. The presence of foreign cells in the tissues of the irradiated mice was detected by the staining properties of the cells, their immunological properties, or by marker chromosomes (*6–12*). The injected cells produced erythrocytes and repopulated the marrow and red pulp of the spleen (*6, 7, 10, 11*) and under some conditions repopulated the thymus and lymph nodes (*8, 9, 12*).

In the experiments to be reported here the presence of homologous bone-marrow cells in the tissues of the irradiated, treated mice was determined by the capacity of these tissues to initiate immunity when injected into non-irradiated mice of the same strain as the irradiated ones. When immunity developed in these test mice, a nonvascularized homograft, which had been previously placed in each mouse, disintegrated. A nonvascularized homograft does not initiate immunity (*13*) but if a homograft, which becomes vascularized, is placed at some other site or if an injection of homologous cells is made, the host becomes immune and the graft disintegrates. The homografts in the test mice were obtained from mice of

[1] Received for publication May 20, 1957.

[2] Homologous tissues are from genotypically different animals of the same species, whereas heterologous tissues are from animals of different species.

[3] The authors would like to acknowledge the assistance of Mrs. Mary W. Fox of the Biometry Branch, National Cancer Institute.

[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[5] Operated by Union Carbide Nuclear Company for the U. S. Atomic Energy Commission.

the same strain as the donors of the protecting bone marrow. Thus, when homologous cells were present in the tissues of the irradiated, treated mice, the test mice developed immunity to the homologous tissue and the nonvascularized homografts disintegrated. During the first 4 days after treatment a few homologous cells were present in the bone marrow. In 12 to 14 days many more homologous cells had appeared. At 30 days and also at 100 days the lymph nodes, spleen, and thymus as well as the marrow contained many homologous cells.

A comparison of the antigenicity, in test mice, of known amounts of tissue from irradiated recipient mice was made with known amounts of tissue from normal mice of the donor strain. This comparison allowed us to make a rough estimate of the amount of homologous tissue in the tissues of the treated mice. The antigenicity was measured by: 1) the number of test mice in which the grafts disintegrated as compared with the number in which they did not disintegrate; 2) the length of the interval observed after the injection of the tissues before the disintegration of the nonvascularized homografts.

These comparisons were based on two assumptions. First, it was assumed that cells in normal marrow had approximately the same antigenicity as cells in marrow of the treated mice, though the proportions of various cell types may have been different. Second, it was assumed that homologous cells in the irradiated mice had not changed significantly in their antigenicity, though it has been shown that a foreign environment may change cells immunologically (*14*, *15*).

Materials and Methods

Four strains of mice or F_1 hybrids were used: C3H, C, (C × A)F_1, and (L × A)F_1. These strains were of the following substrains, respectively: C3H/HeN, BALB/cAnN, (BALB/cAnN × A/LN)F_1, and (C57L/HeN × A/HeN)F_1 (*16*). The bone-marrow donors were C3H mice. The irradiated recipients were (L × A)F_1, (C × A)F_1, or C mice.

Mice, 3 to 4 months of age, were irradiated with 900 r of X rays to the whole body. The mice received 93 r per minute from 2 oppositely placed tubes whose foci were 54 cm. from the center of the mice. The irradiation conditions were: 186 kvp and 20 ma., 0.25 mm. Cu and 1.06 mm. Al filters.

Within 4 hours after irradiation, the marrow obtained from 1 femur of a C3H mouse and suspended in 0.5 ml. of Tyrode's solution was injected into the tail vein of each irradiated mouse. The marrow donors ranged in age from 2 to 4 months.

At various intervals after treatment, the mice were killed and suspensions of cells from hematopoietic tissues and blood were tested for the presence of homologous cells. The suspending medium for the cells being tested was a tissue-culture fluid containing horse serum, chicken embryo extract, and a balanced salt solution (*17*). For each bone-marrow suspension, the ends of a femur were cut off and the marrow washed out with 0.5 ml. of the tissue-culture fluid. Thymus glands, lymph nodes, or

spleen were macerated in 0.5 ml. of the culture fluid and the pieces of tissue were removed with fine forceps. The suspensions contained almost entirely separate cells except for the spleen cell suspensions in which the erythrocytes were agglutinated by the horse serum.

Various known fractions of the total number of cells obtained from the lymph nodes or other tissues of the treated mice were tested. The original suspension, which contained all the cells from any one tissue, was diluted until 0.5 ml. of the culture fluid contained the desired fraction. In a few experiments, the suspension of cells from normal C3H mice was diluted until it contained the same number of cells in 0.5 ml. of the culture fluid as the suspension of cells from the treated mice.

A hemocytometer was used to count the number of cells in some of these suspensions; both erythrocytes and leukocytes were counted.

Cell suspensions to be tested for the presence of homologous cells were injected subcutaneously into test mice, which ranged in age from 3 to 10 months. Each of these mice carried a nonvascularized homograft of harderian-gland tissue transplanted 1 week to several months previously (*13*). Harderian-gland tissue, obtained from C3H mice less than 3 days old, was used because it contains large pigment cells whose disintegration can be detected easily. Each graft was placed under the skin on one side of the test mouse, and a glass coverslip, to serve as a window, was placed under an opening in the skin on the other side. Every 2 or 3 days the skin was pulled up along the mid-dorsal line so that the graft could be observed through the window (*13*). The time of disintegration of the graft was recorded. If the graft had not disintegrated by 30 days, the result of the test was considered negative since disintegration rarely took place after 30 days.

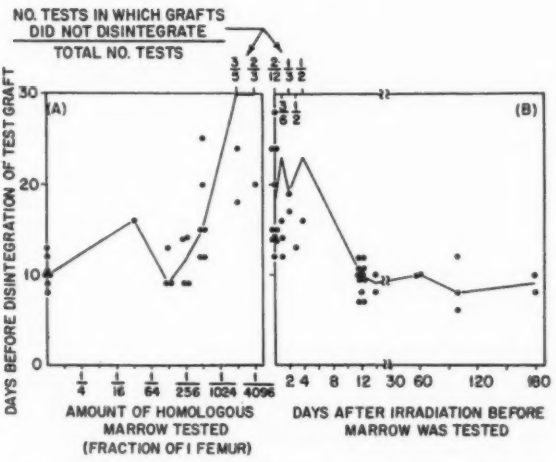
Autopsies were performed on many of the irradiated, treated mice at the time when tissues were taken for the harderian-gland test. Samples of the tissues tested were prepared for histological study. Sections were stained with hematoxylin and eosin.

Results

Tests for Presence and Quantity of Homologous Tissue in Marrow of Irradiated Mice Injected with Homologous Marrow

Tests up to 4 days after irradiation and treatment showed that each suspension containing the marrow from 1 femur of a treated $(L \times A)F_1$ mouse was, on the average, as antigenic as each suspension containing 1/2048 to 1/512 of the marrow from 1 femur of a normal C3H mouse (text-fig. 1).

Twelve days after treatment the marrow tissue was more antigenic than up to 4 days after treatment, and it still maintained this increased antigenicity at 180 days. The marrow from each femur was as antigenic as 1/256 to all the marrow from any 1 femur from a normal C3H mouse. These amounts of normal C3H marrow, ranging from 1/256 to all the femoral marrow, however, caused approximately the same response. Therefore, in order to obtain a closer estimate of the amount of homologous


NO. TESTS IN WHICH GRAFTS DID NOT DISINTEGRATE
TOTAL NO. TESTS
DAYS BEFORE DISINTEGRATION OF TEST GRAFT
(A)
(B)
AMOUNT OF HOMOLOGOUS MARROW TESTED (FRACTION OF 1 FEMUR)
DAYS AFTER IRRADIATION BEFORE MARROW WAS TESTED

TEXT-FIGURE 1.—Tests to determine how much donor homologous tissue was present in the femoral bone marrow of irradiated recipient mice. A.—Tests on marrow from normal mice of the donor strain (C3H). B.—Tests on marrow from irradiated recipient mice (L × A)F_1. Lines are drawn between the median points. Tests on the entire marrow contents of femurs of C3H mice were made on 1 femur from each mouse. The 23 tests on fractions of the femoral-marrow content were made on 9 femurs from 7 mice. The marrow from each femur of each mouse was tested 0 to 14 days after treatment, but the marrow from only 1 femur was tested at 30 to 180 days.

tissue present in the treated animals, it was necessary to compare the antigenicity of fractions of the marrow content of femurs from treated mice with fractions from normal mice.

At 30 and 60 days after treatment, fractions of the marrow content of the femurs from treated mice were approximately one fourth to equally as antigenic as similar fractions from normal C3H mice (table 1). Further, it was estimated that one eighth to all of the marrow cells from the treated mice were homologous since the suspension prepared from 1 of the femurs of a treated mouse contained approximately 1½ times the average number of cells contained in suspensions prepared from the femoral marrow of normal C3H mice. The suspension of marrow from the treated mouse contained 38,000 ± 3,800 cells in 0.5 ml. of suspending fluid at a dilution of 1:1024. Ten suspensions of marrow from the femurs of normal C3H mice, similarly diluted, contained on the average 25,500 ± 3,600 cells with a range from 20,000 to 40,000 cells.

The findings at 100 days indicated that many homologous cells were still present in the marrow of the treated mice.

Tests for Presence and Quantity of Homologous Cells in Blood and in Hematopoietic Tissues of Irradiated Recipient Mice

In all except 1 of the tests on 0.05 ml. of blood taken from mice during the first 14 days after treatment, the nonvascularized harderian-gland

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TABLE 1.—*Results of harderian-gland test for the presence of homologous (C3H) cells in the hematopoietic tissues from mice that had been irradiated and injected with homologous (C3H) marrow*

No. mice tested*	Strain of mice tested	Time after irradiation (*days*)	Tissue tested	Fractions of tissue tested					
				1/128	1/256	1/512	1/1024	1/2048	1/4096
				Number of tests in which the harderian-gland grafts showed early† (E), late (L), or no (N) disintegration					
				E–L–N	E–L–N	E–L–N	E–L–N	E–L–N	E–L–N
2	C3H	No irrad.	Marrow	3–1–1	2–1–1	0–0–4	0–0–5		
2	C	30	Marrow	2–3–1	1–1–4	1–1–5	0–0–4		
			Lymph nodes	1–0–2	0–1–2	0–0–2			
			Spleen				0–1–1		
2	C3H	No irrad.	Marrow			3–0–0	2–1–1	1–0–3	0–1–4
			Marrow				2–0–3	0–1–1	
2	(C × A)F_1	60	Thymus	1–0–1		1–0–1			
			Lymph nodes		3–0–0		2–0–3		
			Spleen				1–0–0	2–0–0	
1	C3H	No irrad.	Marrow			0–2–3‡			
			Thymus					3–0–2‖	
			Marrow			1–0–4‡§			
1	(C × A)F_1	100	Thymus	4–1–0	2–1–2‖				
			Lymph nodes		1–0–0				
			Spleen					1–0–0	

*Tests were made on the marrow from each of the femurs of these mice.
†Early disintegration took place in 6 to 17 days, and late disintegration, 18 to 30 days after injection of the tissue being tested.
‡Both of these bone-marrow suspensions contained about 72,000 cells per 0.5 ml.
§Fractions used were 1/384 rather than 1/512.
‖Both of these thymus cell suspensions contained about 94,000 cells per 0.5 ml.

homografts in the test mice did not disintegrate and the test was considered negative. In 30 days or more after treatment, all the tests, except 1 at 30 days, were positive (table 2). Tests on hematopoietic tissues up to 14 days after treatment could not have been positive because of the blood content of the tissues, except for tests on spleen. The suspensions prepared from spleen contained more than the number of erythrocytes found in 0.05 ml. of blood, whereas the suspensions prepared from the other tissues contained only a small fraction of this amount. By 30 to 180 days after treatment, when the blood gave a positive test, the suspensions of thymus cells prepared from the entire organ may have contained enough blood cells to affect the test, but the fractions of the suspensions prepared from the various hematopoietic tissues did not contain enough blood to affect it. Thymus suspensions prepared from the whole organ contained less than approximately 16,000,000 erythrocytes. The fractions of sus-

TABLE 2.—*Number of irradiated recipient mice whose hematopoietic tissues caused disintegration of the harderian gland (+ test)*

Tissue tested*		Number of + harderian-gland tests / Total number of tests at indicated time after irradiation			
Kind	Amount	0–4 days	12–15 days	30 days	60–180 days
Bone marrow	1 femur	13/14	7/7		
	1/128 or less			2/2	5/5
Lymph nodes	1/3 to all	1/2	2/2		
	1/128 or less	—	—	1/1	3/3
Thymus	3/4 to whole	0/2	2/2	2/2	
	1/128 or less	—	—	—	2/2
Spleen	3/4 to whole	2/3	3/3		
	1/1024 or less	—	—	1/1	3/3
Blood	1/20 ml.	0/5	1/2	0/1	7/7

*The irradiated recipient mice were $(L \times A)F_1$, $(C \times A)F_1$, and C. The donor bone marrow came from C3H mice.

pensions prepared from the various hematopoietic tissues contained less than approximately 900,000 erythrocytes. Twelve tests were negative on blood from C3H mice diluted until 0.5 ml. of the suspending fluid contained approximately 1,700,000 to 7,000,000 erythrocytes. Only 3 out of 9 tests were positive when a volume of blood containing approximately 28,000,000 to 56,000,000 erythrocytes was injected.

Tests on marrow, spleen, thymus, and lymph nodes (table 2) indicated that the spleen and lymph nodes, like the marrow, contained a few homologous cells up to 4 days after irradiation and that, like the marrow, these tissues and thymus showed an increased antigenicity in 12 to 14 days after treatment. Furthermore, except when very small fractions were used, all these tissues, like the marrow, were antigenic in every test made 12 to 180 days after treatment.

At 60 days after treatment, a suspension prepared from 9 pooled lymph

nodes contained approximately 15,000 ± 850 cells per 0.5 ml. of suspending fluid at a dilution of 1:1024. This suspension was approximately as antigenic as suspensions, at the same dilution, of femoral marrow cells from normal C3H mice. The average number of cells present in suspensions, each of which was prepared from the marrow of 1 femur of a C3H mouse, was 25,000 ± 3,600 cells at this dilution. Thus, unless the lymph-node cells were more antigenic than the marrow cells, approximately one fourth to all of the lymph-node cells were homologous.

The results of tests on thymus suspensions 60 days after treatment indicated that many homologous cells were present. Tests at 100 days on suspensions containing equal numbers of cells from either a normal C3H mouse or from a treated mouse indicated that one fourth to all the cells from the thymus of the treated mouse were homologous. A sample of the thymus of the treated mouse studied histologically showed atrophy, though cortical tissue was present. The lymph nodes also showed atrophy, whereas the white pulp of the spleen appeared normal.

Pathologic Findings in Hematopoietic Tissues of Irradiated Recipient Mice

In mice killed and autopsied at 60 or 100 days after treatment, the lymph nodes were reduced one fourth to one half the size of that seen in normal mice. The thymus was about half and the spleen about twice the size of that seen in normal mice.

The bone marrow attained normal cellularity within 12 days after irradiation and remained this way, or nearly so, at later intervals. The time of repopulation of the marrow coincided with the time of increase in number of homologous cells as indicated by the results of the harderian-gland tests.

After irradiation, the red pulp of the spleen showed hyperplasia of the blood-forming cells from the 12th to the 60th day. At later intervals, the blood-forming cells in the red pulp were present in about normal amounts. The white pulp was atrophic in all the pieces of spleen taken 30 days or less after treatment. This atrophy resembled that seen in the delayed homograft bone-marrow reaction reported by Congdon and Urso (*18*). By 60 days, the white pulp was atrophic in 1 spleen and slightly atrophic in another. By 90 to 180 days it was normal.

During the first 30 days after irradiation and injection with homologous bone marrow, the lymph nodes showed atrophy, granulopoiesis, and reactive changes just as those described by Congdon and Urso (*18*). In the present experiments, in 60 to 180 days after treatment, no granulopoiesis was observed and, although the lymph nodes were still atrophic in some mice including 2 whose lymph nodes gave a positive test, others contained lymphocytes and showed lymphopoiesis in varying degrees. A representative lymph node, from the animal that was killed and autopsied at 180 days after irradiation, appeared normal in architecture.

From these histological findings on the lymph nodes, it seems possible that the positive tests, obtained at 30 days and earlier, were due to

homologous granulocytes rather than to lymphocytes. Although no granulopoiesis was found in lymph nodes 60 days or more after treatment, granulocytes may have been present in the pooled suspensions. Therefore, one cannot exclude the possibility that, even at 60 and 100 days after treatment, the positive tests resulted from granulocytes rather than from lymphocytes.

It is known that a temporary regeneration of the thymus occurs in irradiated mice that have been given homologous bone marrow (*18*), but a secondary atrophy usually follows during the 4th week. In this atrophy the normal architecture of the gland is sometimes preserved. In the mice studied here, regeneration was observed in 12 to 15 days. In 95 to 108 days the thymus showed either atrophy with cortex present or normal architecture. Since granulopoiesis was observed in 1 thymus at 12 days after treatment, it must be considered that the positive tests for thymus may have been due to the presence of granulocytes.

Discussion

Our observations that homologous cells appeared in the marrow of the irradiated, treated mice on the day of injection and that these homologous cells increased markedly in 12 to 14 days agree with the findings of Nowell *et al.* (*7*). The results reported in the present paper indicate that at 30 and 60 days after treatment the marrow of the treated mice contained approximately one eighth to all homologous cells. Other studies in which heterologous or homologous spleen or bone marrow was used for protection indicate that all the erythrocytes and granulocytes are derived from the injected cells (*8–11*). Lindsley *et al.* (*6*), however, found that under the conditions of their experiments various proportions of the numbers of erythrocytes were of the host type.

The lymph nodes and thymus glands have been described here as containing many homologous cells. The lymph nodes at 60 days and a thymus at 100 days were estimated to have between one fourth to all homologous cells. Although the homologous cells found before 60 days may have been granulocytes, it does not seem likely that those present in 60 to 100 days were granulocytes. Mitchison (*12*) recorded that donor antibody-producing cells were active in the irradiated, treated recipients. Also Ford (*8*, *9*) found that even at 100 days after injection of spleen all the cells undergoing mitosis in the thymus and lymph nodes were of the donor type. Ford did not mention the presence of granulopoiesis in the thymus or lymph nodes but it is unlikely that all cells undergoing mitosis at 100 days after treatment could have been granulocytes. Although these observations of Mitchison and Ford indicate that spleen can produce lymphocytes, yet Makinodan (*10*) and Gengozian and Makinodan (*19*) concluded that neither isologous nor heterologous marrow injected into irradiated mice contributed to the antibody-forming tissues of the host—suggesting that the marrow does not form lymphocytes. Although the present observations suggest that marrow does form lymphocytes, more work is needed to prove whether it does or does not.

If lymphatic tissue of the irradiated host regenerated, it might result in passive transmission of immunity by the suspensions obtained from the spleen, lymph nodes, or thymus. However, a large volume of tissue is needed to obtain passive transmission (*20, 21*) and, therefore, only the undiluted suspensions tested at 12 or 30 days could be effective. The harderian-gland test has been found (unpublished work) to be no more sensitive to passive transmission than are other tests.

Summary and Conclusions

Mice were irradiated with 900 r to the whole body, and then each was given an intravenous injection of a suspension of marrow cells from 1 femur of a mouse of another strain (C3H). Tests were made for homologous (C3H) cells in the marrow, blood, spleen, lymph nodes, and thymus of the irradiated recipients. A sufficient number of homologous bone-marrow cells initiated immunity to homologous tissue when injected into test mice. Immunity in the test mice was detected by the disintegration of a nonvascularized C3H homograft that had previously been placed in each test mouse.

During the first 4 days after irradiation and the injection of homologous C3H marrow, each femur contained a small number of homologous cells. Twelve to 14 days after irradiation, the marrow had regained its normal cellularity and the antigenicity had increased. In 30 to 60 days each femoral marrow from the treated mice contained C3H tissue equivalent to from one fourth to the total marrow content of a femur from a normal C3H mouse. These results, and those of others, demonstrate that the marrow of mice irradiated and given marrow or spleen is repopulated by the donor cells.

The spleen and lymph nodes, like the marrow, gave some positive tests after treatment up to 4 days. These tissues and the thymus gave consistently positive tests by 12 days. Blood, in the amount used, did not give consistently positive tests until after 30 days. The thymus glands and lymph nodes in 60 to 100 days contained many homologous cells. At 100 days the thymus of 1 animal contained approximately one fourth to all homologous cells. Although it has been indicated by the work of others that injected spleen repopulates the lymph nodes and thymus of irradiated hosts, this is not yet proved for marrow injections. The findings reported here suggest that the thymus and lymph nodes are repopulated by lymphocytes derived from the donor; however, the evidence is not conclusive because of the possibility that the positive tests were due to homologous granulocytes rather than lymphocytes.

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The Adaptation of Additional Lines of NCTC Clone 929 (Strain L) Cells to Chemically Defined Protein-Free Medium NCTC 109 [1,2]

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The development of chemically defined, protein-free media in which animal tissue cells can be routinely and indefinitely cultivated has received much attention in recent years. These media would make possible the establishment and maintenance of cell strains that are free from the effects of unknown factors and inherent variations of complex proteinic materials which occur in sera, dialyzed serum residues, embryo extracts, or incompletely defined protein hydrolysates. Cell strains well adapted to and stabilized by maintenance in a chemically defined medium would, in turn, make possible more highly controlled and reproducible experimentation *in vitro* in such fields as cell nutrition, carcinogenesis, and chemotherapy.

Evans and associates (*1*, *2*) have developed an entirely chemically defined, protein-free medium, designated as medium NCTC 109.[4] This has been used to establish and maintain reliably a strain of mouse cells from NCTC clone 929 (strain L) [5] in continuous and reasonably rapid cell proliferation for more than 2 years up to date. Proliferation of the cells in

[1] Received for publication May 21, 1957.

[2] Presented in part at the Eighth Annual Meeting of the Tissue Culture Association in Baltimore, Md., April 17, 1957.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[4] The papers cited describe media NCTC 107 and 108. The modifications of the former medium for producing NCTC 109 are given in the Materials and Methods section following.

[5] Because some confusion exists with respect to the identification of this cell strain during the 16 years or more it has been under study in this laboratory, its history is briefly recapitulated here.

The original parent strain, designated 202, was taken from the subcutaneous and adipose connective tissue of a C3H strain, Andervont substrain, 100-day-old male mouse, October 18, 1940 (*3*). One group of cultures from this parent strain was treated with 3-methylcholanthrene at a concentration of 2.0 to 2.5 γ per cc. for 111 days and was designated strain L. Cultures of this strain developed the capacity to initiate a large percentage of sarcomas upon reinjection into its original parent mouse strain (*4*). This ability, however, was never demonstrated as arising from treatment with methylcholanthrene, and it steadily deteriorated over a period of years until it became very low (*5*).

Strain L was cloned by single-cell capillary isolation in 1948 (*6*) and was designated clone 929. All cultures released from this laboratory with the strain identification 929 or clone 929 have come from this clone. Cells of this clone continued to show only a very low percentage of sarcomas when cultures were reinjected into the original mouse strain (*7*). In all of this earlier work the cells were maintained in chick-embryo extract : horse serum : balanced saline nutrient medium.

In order to avoid confusion with cell strains from other sources, cell strains from the Tissue Culture Section, Laboratory of Biology, National Cancer Institute now have the identifying letters NCTC before the strain number, and the current designation of the strain in the present study is NCTC clone 929. The substrain established and maintained in medium NCTC 109 for more than 2 years is now considered an established strain and is designated NCTC strain 2071. In this paper the prefixed letters will be omitted in further reference to these strains.

this medium during the last year has been sufficiently rapid to allow weekly serial subculture. This established strain, now routinely maintained in T-60 flasks and designated as NCTC strain 2071, is currently being used in this laboratory in further nutrition and metabolism studies.

The original adaptation of cells of clone 929, from a nutrient medium rich in proteins to the chemically defined, protein-free medium, required careful nurturing for a period of 6 months before there was assurance that the cultures would continue to proliferate rapidly and could be readily subcultured and thus considered stabilized. It therefore seemed desirable to attempt the adaptation of additional cultures of clone 929 cells to medium NCTC 109 in order to study in more detail the adaptation phenomenon and the efficacy of this medium for long-term tissue cultures. These studies are reported in this paper.

As used herein, the term "adaptation" refers to whatever modifications are undergone by the individual cells and/or the cellular population as a whole, in the process of becoming better able to live in the new nutritional environment.

The term "critical period" is used to designate the period of time during which the cultures undergo visible changes in the process of attempting to adapt to the new medium. This period may be mild or severe, short or extended. It is characterized by changes in cell morphology and by a decline in cell-population growth rate. For cultures that succeed in adapting, the critical period is considered at an end when the morphology and growth rate appear to have stabilized and the cultures can be serially transferred at weekly intervals like cultures of the progenitor clone 929.

Materials and Methods

The preparation and components of medium NCTC 107 have been previously described in detail (*1*). Chemically defined, protein-free culture medium NCTC 109, used in the present work, is identical with NCTC 107 except for the following changes: 1) vitamin B_{12} is added at a concentration of 0.01 mg. per ml.; 2) the 3 unsaturated fatty acids and the cholesterol are omitted; 3) the amount of Tween 80 is reduced to 1.25 mg. per 100 ml.

All experiments were begun with established cultures of clone 929 cells growing in either T-60, T-15, or Carrel D-3.5 flasks. In two instances, experiments 2340 and 2454, the originating cell line had been maintained for the preceding year in NCTC 109 supplemented with 1/4 percent horse serum; in all other experiments, stock-culture medium, composed of 20 percent filtered chick-embryo extract (*8*), 30 percent pooled horse serum, and 50 percent Earle's balanced saline, had been the former medium. At the start of each experiment, either the stock medium or NCTC 109 supplemented with serum was withdrawn from the well-established cultures and was replaced with NCTC 109, except in experiment 2347 in which stock medium was first replaced by NCTC 109 supplemented with 1/4 percent horse serum for a short transitional period.

With minor exceptions, fluid renewals and gassing of cultures with

5 percent CO_2 in air, were made thrice weekly. Cell subculturing, followed by gassing, was done as the cell populations appeared to warrant, usually determined by the crowding of cells on the glass substrate. For subculturing, cells were removed from the floor of the flasks by pipette flushing, platinum-rake scraping, and later, by cellophane strip mopping; flask shaking was found to be inadequate to remove the cells from the glass because of the tendency of cells in NCTC 109 to adhere tightly to the substrate. The resultant cell suspension was usually divided equally between 2 fresh daughter cultures. This type of cell transfer is referred to as a 1-to-2 subculture. Occasionally, all cell suspension from a flask was subcultured to a single fresh flask, and this type of cell transfer is designated as a 1-to-1 subculture. The latter procedure was used to effect subculturing without undue reduction of cell population in the resultant daughter culture, to attain more even cell distribution over the glass, or to eliminate accumulated cellular debris. Each serial subculture of a parent to one or more daughter cultures has been considered a culture generation.

To avoid maintaining an excessive number of cultures, those not used for cell transfer, including many good cultures, were generally discarded a few days after new daughter cultures were established and proliferating. This stringent discarding of cultures was later modified to allow a longer period of culture observation and provide a larger number of cultures from which to make selections.

Frequent detailed microscopic observations, written records, and photomicrographs were made of individual cultures.

Experiments and Results

Adaptation Experiments

Thirteen experiments were set up in an attempt to adapt 56 separate cell lines of clone 929 cells to medium NCTC 109. Criteria of successful adaptation of a cell line to this synthetic medium were: 1) cessation of recognizable cellular degeneration; 2) attainment of a relatively rapid proliferation rate which allowed weekly serial subculture; and 3) survival for a minimum of 100 days and at least 10 transfer generations in such condition that the cultures could be routinely maintained without reasonable doubt as to their ability to survive indefinitely. One exception to this was made in the early phase of the study when experiment 2237 was closed as successfully adapted at 62 days.

Adapted lines were arbitrarily terminated soon after the above criteria were met. Culture lines that failed to adapt to the new environment were maintained as long as there appeared to be any reasonable possibility of their survival. They were discontinued only when the last culture of each line contained no live cells or only a very scant population of necrotic or abnormal-appearing cells, with an apparent cessation of proliferation.

Results of these adaptation experiments are summarized in table 1,

TABLE 1.—*Data on cell lines of NCTC clone 929 grown in chemically defined, protein-free medium NCTC 109*

Experiment no.	Number of initial cultures	Number of attempts to establish cell lines			Range of survival or arbitrary termination time of cell lines	
		Total	Succeeded	Failed	Days	Generations
2183	1	1	0	1	88	5
2216	1	1	0	1	11	2
2237*	10	10	1	9	26–62	2–5
2261	8	8	0	8	35–45	3–4
2299	10	10	0	10	25–53	2–3
2340	1	1	1	0	148	17
2347	2	1	1	0	187	14
2348	4	4	1	3†	60–133	5–13
2349	15	15	1	14†	25–283	1–24
2430	1	1	1	0	135	15
2454	4	1	1	0	103	15
2468	4	1	1	0	107	10
2519	8	2	2	0	131	11–14
TOTAL	69	56	10	46†		

*This experiment was conducted before the more rigid criteria of survival for a minimum of 100 days and 10 transfer generations were adopted.

†Four cell lines were lost in laboratory accidents.

and detailed descriptions of some of the most significant experiments are given in the following sections.

Experiment 2340.—One T-60 culture of cells that had been maintained for the preceding year in NCTC 109 supplemented with 1/4 percent horse serum was shifted to NCTC 109 alone. For the first several weeks, large, bizarre cells with numerous extremely long processes were outstanding in some cultures and even more exaggerated than those in figure 8. On the 50th day, the seventh weekly serial 1-to-2 subculture was made. During the following 5 weeks the proliferation rate decreased somewhat and allowed only 3 serial subcultures. Two eleventh-generation cultures appeared to be in good condition at 3 days but continued the slow growth rate. One of these was subcultured at 2 weeks of age to a single daughter culture. At 5 days only a few scattered live cells remained in this culture, and it was discarded when the cell line had been in NCTC 109 for 104 days. The other eleventh-generation culture had cells in good condition at this time but only in about one fifth of the flask area. Cells of this limited area began to proliferate well, however, and during the following week numerous other growing centers arose from small cell masses that sloughed off the one healthy area. Six T-9 flask cultures were made from this one 26-day-old T-60 culture. Five days later, cells of each T-9 culture were subcultured 1-to-1 to T-15 flasks. These, and subsequent daughter cultures, appeared to be completely adapted to the chemically defined medium. Proliferation was rapid and cell morphology appeared reasonably stable and uniform with an even and dense cell distribution over the flasks comparable to cells of figure 19. Four 1-to-2 serial subcultures were made in the next 24 days. The experiment was terminated as a successful adaptation after 148 days and 17 generations.

Experiment 2347.—Two T-60 flasks of clone 929 cultures were first started in NCTC 109 supplemented with 1/4 percent horse serum in an attempt to adapt gradually an individual cell line to chemically defined medium by diluting out the serum. At 19 days, third-generation cultures appeared to be in good condition, and 2 cultures were put in NCTC 109 alone. Four successive weekly 1-to-2 subcultures were easily made. The single retained seventh-generation culture, however, degenerated completely within

25 days, although its population and cellular condition at 1 day of age had seemed to justify discarding its sister culture as not being needed to maintain the cell line.

Another culture that appeared well adapted to the dilute horse-serum medium at 5 generations and 35 days meanwhile had been put in NCTC 109. Two serial subcultures were made in 2 weeks. Although the 2 resulting seventh-generation cultures appeared in good condition for the first 3 weeks, they degenerated markedly thereafter in both the proliferation rate and the condition of cells. One culture was lost by a laboratory accident; the other T-60 culture, containing a few small areas of healthy cells in a generally very poor culture, was subcultured at 55 days of age to 2 T-15 flasks. One of these 2 cultures died within a week; the surviving culture continued in poor condition. At 16 days this culture of the fourth generation in NCTC 109 alone showed only a single small area of healthy cells on a surface otherwise covered with cell debris. This culture was followed closely both microscopically and by frequent photographs (figs. 1–6). Several fluid renewals were omitted at different times or only partial renewal of the medium was made when observations of the cell population and of the *p*H of the medium suggested that cell metabolism and proliferation rate were low.

The cells at the periphery of the growing area of the surviving culture appeared to be less healthy than those at the center and appeared to have difficulty in increasing the area of cell growth either by migration or proliferation. The population in the center, however, became dense, and from this dense core cells and small cell clumps sloughed off and reimplanted in other areas of the flask, where they continued to proliferate. This redistribution of healthy cells was aided at 27 days by gently shaking the flask. These new discrete centers of growth gradually increased in number and population density for the next 2 weeks. When the culture was subcultured 1-to-1 at 43 days, an even distribution of rapidly proliferating cells resulted. The cell line appeared to have completely adapted to the protein-free medium after an extended critical period of 14 weeks comprising only 2 culture generations. No difficulty was experienced thereafter with 1 biweekly and 7 weekly 1-to-2 subcultures. The experiment was terminated when it had been on NCTC 109 with no serum supplement for 187 days and 14 generations.

Experiment 2348.—Four cell lines were started, each as 1 Carrel D-3.5 flask culture. On 2 of the cultures, the B set, NCTC 109, conditioned by 1-day use on cultures of strain 2071 and centrifuged to eliminate cells, was used instead of fresh medium. The 2 cultures of set A received fresh NCTC 109. For the first 13 days, set B continued to receive conditioned fluid or to have only partial fluid renewals. The total quantity of fluid in all flasks, however, was maintained at the same level. In 2 weeks, a flask of each set was subcultured to the second generation. No significant difference could be determined between the sets as a result of the 2 different treatments; therefore complete fluid renewals with fresh medium were instituted in set B.

One cell line was lost by a laboratory accident; 2 others were carried into the fifth generation but were discarded at 60 and 96 days, respectively, when practically no live cells remained. Five days prior to being discarded 1 of these cultures had been observed to contain a small area of viable cells like that which gave rise to an adapted cell line in experiment 2347.

The remaining line of set A successfully adapted to NCTC 109 and was discontinued after 133 days and 13 generations in the chemically defined medium (figs. 7–10). Although these cells underwent morphologic and population changes like those observed in other experiments, the critical period was not as severe as in some other instances. Cells were subcultured progressively from Carrel D-3.5 to T-9 and to T-15 flasks. The last four 1-to-2 subcultures were made at weekly intervals.

A subline, descended from the same original culture that gave rise to the successful line of set A, had a history of good proliferation with 4 successive subcultures in a period of 35 days. Thereafter, however, 2 sixth-generation cultures showed gradual deterioration which progressively became so severe that they were discarded within 1 month (figs. 11–14).

Experiment 2349.—Fifteen Carrel D-3.5 flask cultures were used to originate 15

parallel cell lines on NCTC 109. Three of these original cultures degenerated within the first few weeks without having developed adequately for subculture. Three other lines were lost as a result of a laboratory accident.

Of the remaining 9 lines, which were all subcultured to T-9 flasks, 7 were carried to the sixth or seventh generation at 41 days in NCTC 109 and appeared to be in good condition for a few days thereafter. However, within a week, microscopic examination of the cultures revealed cell degeneration and retarded proliferation in varying degrees. Subcultures were made during the following month as individual cultures appeared to warrant, but each line gradually or abruptly completely degenerated. All 7 lines were discarded in the sixth to eighth generation by the time the cultures had been in chemically defined medium for 76 days.

The final 2 lines appeared for a time to have adapted to the new medium. However, the onset of the critical period for these lines merely occurred several weeks later than for the other 7 lines. The same degenerative pattern was repeated and accompanied by gradual cell-population decrease in spite of several 1-to-1 subcultures. One of these 2 remaining lines was discarded as unsuccessful in the ninth generation at 102 days.

Although in poor condition, the final line was surviving at 112 days in the tenth generation in a single T-15 culture flask. At 13 days, only scattered viable cells were observed among cellular debris. Two days later, one small discrete area appeared to contain healthy cells capable of proliferating. Within the next 56 days the number of cells in this 1 small area gradually became sufficiently dense to cause cells and small cell clumps to slough off, reimplant in other areas of the flask, and continue to proliferate. This dissemination of healthy cells, assisted by gentle shaking of the flask, ultimately resulted in fair cell growth throughout the flask. Five serial subcultures, 4 of which were in the conservative 1-to-1 manner, brought the cell line to the fifteenth generation during the next 34 days. Although the cultures did not appear completely adapted to the medium during this period, they were gradually improving. The predominance of stellate cells, frequently observed in cultures in the adaptive process, was apparent (fig. 15), and the cell population increased. Following this protracted critical period of approximately 21 weeks, the line appeared completely adapted, and 9 weekly 1-to-2 subcultures were accomplished with no further difficulties until the successfully adapted line was terminated in the twenty-fourth generation at 283 days in NCTC 109. The cells were like those in figure 19.

Experiment 2468.—The procedure here was to adapt a single line rather than separate lines from 4 original T-15 cultures. Four to 6 cultures were usually maintained at any one time. After the first 2 weeks, during which time the cultures appeared healthy with a predominance of long, slender, fusiform cells, comparable to those of figure 12, the population growth rate declined and an increased proportion of large, multiprocessed cells, comparable to those of figures 2 and 8, were observed. The cultures appeared to undergo a comparatively mild but extended critical period, during which time 3 serial 1-to-2 subcultures were made at 3-, 2-, and 4-week intervals, respectively. Thereafter, the cultures appeared to be completely adapted to the new medium. Population growth warranted 4 successive weekly 1-to-2 transfers. The experiment was terminated as a successful adaptation when the cultures had been maintained for 10 generations and 107 days in NCTC 109.

Readaptation Experiments

Two lines of strain 2071 cells were used to determine the effect on cultures when they were returned to protein-containing medium and then again returned to chemically defined medium after having been originally adapted to and maintained in chemically defined NCTC 109 for an extended period of time.

Experiment 2380.—At the time strain 2071 had been maintained in chemically defined medium for 14 months, 2 T-9 cultures were returned to stock protein-containing

medium. Cultures were serially subcultured with no difficulty, at weekly intervals, in the 1-to-2 manner for 10 generations in 73 days. During this time the cultures acquired and maintained an appearance somewhat different from that of either strain 2071 cultures, maintained in NCTC 109, or clone 929 cultures, continuously maintained in stock medium. An increase in cell-population density was readily observed when cultures were returned to the stock medium.

The cells of these readapted cultures had a greater tendency toward cohesiveness than had previously been observed among the cells in either medium. This tendency was exhibited microscopically by the appearance of clumps of dense cell growth scattered throughout cultures that appeared to be growing as continuous, cohesive cell layers. Macroscopically, a grainy appearance superimposed on a dense cell layer was observed, and a flocculent appearance resulted when the cells were suspended in the fluid medium for subculturing. The cells showed great variation in size and giant cells frequently appeared. However, the readapted cells showed a decrease in the proportion of bizarre shapes often seen in the chemically defined medium. In general, they were comparable to cells shown in figures 18 and 22.

Two of the cultures that had been readapted to the protein medium and maintained therein for 63 days and 9 generations, were returned to NCTC 109. No difficulty arose in this treatment, and the cell line was continued with weekly serial 1-to-2 subcultures to the sixteenth generation and for 57 days, except for one 1-to-1 subculture when the series was changed to the larger T-15 flasks. It was observed that as the cells adapted to chemically defined medium for the second time, the cell appearance again became more nearly like that of strain 2071 (figs. 16 and 20), *i.e.*, cells appeared less compact and showed more processes, and there was a reduction in the number of giant cells and in cell-population density.

Experiment 2557.—When strain 2071 was 19½ months old, 2 T-15 flask cultures of this strain were returned from NCTC 109 to stock medium of embryo extract : horse serum : saline for a second readaptation study. Weekly serial 1-to-2 transfers were easily carried out while the series was maintained for 25 generations and 186 days to date.

When this readapted cell line was in the seventeenth generation and on protein medium for 122 days, 1 T-15 culture, subline 2557A, was subjected to adaptation to chemically defined medium for the second time. Weekly serial 1-to-2 subculturing was routinely continued with no indications of adaptive difficulties.

The same findings reported for experiment 2380 were obtained. However, during the longer time in which the readapted cultures of experiment 2557 (figs. 18 and 22) were maintained in the stock medium, the cells tended to lose some of their exaggerated cohesiveness and wide variability in size and to appear more like the characteristically predominant small compact cells of clone 929 (figs. 17 and 21).

Comparative Population of Cultures

Nuclei enumerations by the procedure of Sanford *et al.* (*9*) were made for the purpose of comparing the cell populations of several lines of clone 929 cultures adapted to NCTC 109, with those of cultures of the original clone maintained in the stock protein-containing medium. Population counts were also obtained for cultures of the readaptation experiment 2557. Representative 9-day-old T-15 cultures with dense, healthy cell growth in about 4 ml. of nutrient fluid were used for this comparison. Cultures of this age were selected because preliminary counts and observations indicated that approximately maximum cell population had been attained but no appreciable detachment from the glass substrate due to overcrowding had yet begun to occur. Normally these cultures would have been subcultured at 7 days of age. Each culture had been derived

from a parent culture by the routine 1-to-2 culture transfer method following suspension of substantially all cells of the parent culture by the cellophane strip mopping technique.

The results of these nuclei enumerations are shown in table 2. They indicate that cell populations of the recently adapted lines 2519 and 2349 were approximately 1/3 that of clone 929 cultures. Long-adapted strain 2071, however, showed a population more than half that of the parent strain. When strain 2071 was readapted to protein medium, line 2557, the population density returned to that of the original cell strain. The population densities of cultures maintained in NCTC 109 for the second time (subline 2557A) again declined but were equal to those of strain 2071 cultures. In summary, these data indicate that the population density level of cultures adapted to and maintained in NCTC 109 is not as high as that of cultures maintained in stock protein-containing medium.

TABLE 2.—*Comparison of cell populations of representative 9-day-old cultures of clone 929 cells in T-15 flasks when given different media treatments*

Cell strain or line	Sequence and time in the 2 different media				Cell population (*in millions*)
	1	2	3	4	
	Protein (*years*)	NCTC 109 (*days*)	Protein (*days*)	NCTC 109 (*days*)	
929	over 15	—	—	—	13. 3*
2519	over 15	107	—	—	4. 5†
2349	over 15	245	—	—	4. 0†
2071	over 15	770	—	—	7. 4*
2557	over 15	595	175	—	13. 3†
2557A	over 15	595	122	53	8. 3*

*Average of 2 cultures.
†One culture.

Discussion

The successful maintenance of strain 2071 for over 2 years and up to date has clearly shown that once the cells of clone 929 have been adapted to the chemically defined, protein-free medium NCTC 109, this medium is able to maintain the cells in a highly proliferative and well-stabilized condition for an apparently indefinite period of time. Similar adaptation has been repeatedly accomplished with other lines of cells from the same clone.

In these adaptations it is believed the time the cells were in chemically defined medium, the number of fluid renewals they received, and the number of subcultures made in each experiment were sufficient to have eliminated the possibility of any dependence of the adapted cell lines upon residual serum from the prior stock medium. Certainly this is the case with the strain maintained in NCTC 109 for more than 2 years.

Although repeated adaptations of clone 929 cells to the chemically defined medium have been accomplished, the adaptive process generally appears to require a radical adjustment by the cells. There is no assur-

ance that every culture or every cell line of this clone, when transferred initially to the chemically defined medium, will succeed in adapting to it, though all cultures appear to grow at least for a limited period of time. Also, during the adaptive process more than usual care must be taken in handling the cultures.

A morphologic change took place in the cells during their process of adapting to protein-free medium after a prolonged life *in vitro* in the protein medium. Frequently, the first apparent morphologic change was that many of the cells became long, slender, and gracefully spindle-shaped to the extent that the morphologic pattern of the culture as a whole was different from that shown in the protein medium (compare fig. 12 with figs. 17 and 21). However, cultures of this pattern did not always subsequently adapt successfully to the new medium. The culture morphology which seemed to be more typical of the critical period of the adaptive process, was that in which many cells assumed a variety of bizarre shapes. The cells appeared larger with numerous processes or had an angular or stubby, fusiform appearance. Cell granularity frequently increased, and numerous necrotic or disintegrating cells were seen (figs. 2, 3, 8, 9, 13, and 14).

During the adaptive process, the cells passed through a critical period in which they either succumbed or adjusted to the new milieu. The time of onset, the severity, and the duration of this stage were all widely variable and unpredictable. Three of the 56 cell lines degenerated from the start and never warranted subculturing, though viability of cells was maintained even in these lines for at least 20 to 30 days. Most of the lines, however, were serially subcultured 1 to 4 times before being terminated as failing to adapt; a few lines persisted through 6 or 9 generations before failing. Of the 10 lines that successfully adapted to the medium, 5 went through very severe and extended critical periods ranging from 4 to 21 weeks; final successful establishment of each of these 5 cell lines was accomplished by the survival and gradual adaptation of a small population of cells in a single culture flask. The other 5 successful lines underwent critical periods of varying duration but of a much milder nature in regard to cell changes and population growth rates, and the ultimately adapted cultures appeared to rise from a much larger proportion of the cell population. The wide variation in the number of days and generations through which cultures of different experiments passed before entering the critical period, and the variability of the severity and duration of this period, may be associated with the variability of the diluting out of the residual proteinic medium or some component thereof in which the cells were originally growing.

In addition to this factor, however, since 9 of the 10 successful adaptations in this study were from the last half of the 56 attempts made, it seems probable that unevaluated minor improvements as well as known gradual changes in techniques contributed to the more successful results of these later experiments. Although no exact correlation can be traced, some of the changes and standardizations in technique, which were made

during the period of the study and which may have contributed to increased success in adaptation, are the following: 1) The chemically defined medium that was formerly made up in 1 and 2 liter batches every several weeks was later made up in much larger lots up to 40 liters and thus afforded greater uniformity of medium over a much longer period of time. 2) CO_2 gassing of cultures, particularly at subculturing, was later done more uniformly and deliberately with 10 to 30 seconds of gassing for each culture to insure that no new culture remained in an over-alkaline medium. 3) Whereas, formerly, cells were suspended for subculture purposes by vigorously shaking the glass and scraping it with a platinum rake, cells were later routinely suspended by being mopped from the glass with a piece of cellophane manipulated by a curved glass rod. 4) T-9, T-15, T-60, and Carrel D-3.5 flasks were used for the different experiments, but the greatest success was obtained when the cultures were either started in or subsequently subcultured to T-15 flasks.

The adhesion to the glass substrate of cells of healthy adapted cultures was much greater in chemically defined medium than that of cells in cultures of comparable age growing in stock medium. The cause of this difference in cell attachment to the glass is not known. In spite of this increased adhesion, however, cell-population density was not as great in the chemically defined medium as in the protein medium. During the adaptive process, population densities of cultures declined; they became stabilized in successfully adapted cell lines at a level about one third to one half that of the parent clone 929 on stock medium. Microscopic observations showed that cells in cultures of clone 929 became much more crowded than those of lines adapted to chemically defined medium. These observations were borne out by nuclei enumerations.

This lower population density associated with cultures in the adaptive process cannot, however, be regarded as the principal or most significant cause of failure of some lines to adapt to the chemically defined medium. It was observed repeatedly that very sparse cultures of clone 929 cells in stock medium and similar sparse cultures of lines that had successfully adapted to NCTC 109, *e.g.*, strain 2071 and line 2349, could survive and proliferate without apparent difficulty. Yet, during the adaptive process, many cultures with relatively large population densities were seen to degenerate gradually over a period of days and weeks; some others failed immediately following subculture.

The cells that successfully adapted are presumed to have undergone some functional changes that enabled them to survive and proliferate in a nutritional medium greatly different from that to which they had been accustomed. The present work does not explore the mechanism of the change that took place. Whether the change resulted from a mutation, a selection process, an enzymatic adaptation, or some combination of them, is not indicated. However, whatever the mechanism, the cells that adapted to the chemically defined medium did become able to satisfy all their nutritional requirements for prolonged life and rapid proliferation from the protein-free medium.

Although the relative rate of cell proliferation in the 2 media has not yet been studied in detail, once a cell line adapted to the chemically defined medium its stability and continued ease of maintenance in the type of static cultures studied appeared to be equal to that of the parent cell strain maintained in stock protein-containing medium. After more than 2 years and 88 generations in NCTC 109, strain 2071 appears to be just as stable in static glass-substrate cultures as clone 929 and is maintained with the same routine procedure. Weekly serial 1-to-2 subcultures are made with equal confidence and reliability. Each of the newly adapted lines showed the same stability after emerging from its critical period and, in fact, was not regarded as successfully adapted until it had demonstrated this stability by rapid proliferation and routine subculturing for several generations.

The functional and morphologic changes that the cells underwent in the adaptive process appeared to be, at least to some degree, reversible. When 2 lines of the adapted strain 2071 (figs. 16 and 20) were returned to stock medium, the cells appeared to have no difficulty. In this return to proteinic medium the cell-population density of the cultures increased and again approached the population density characteristic of cultures of clone 929 cells (*cf.* figs. 21 and 22). There was a tendency for the individual cells to resume the more compact shape of cells of the parent clone, even in uncrowded cultures, except for the frequent occurrence of giant cells during the early weeks of this readaptation to protein medium (*cf.* figs. 17 and 18). Pronounced cellular cohesion was also exhibited. When cultures of these 2 readapted lines were again returned to NCTC 109, there was no indication of difficulty in relinquishing the proteinic medium and no adaptive crisis. Moreover, the population density again decreased and cell appearance again became more like that of cells continuously maintained in NCTC 109.

This ability of cells to return to their former medium of embryo extract : horse serum : saline, after being cultured for a time in a predominantly chemically defined medium, has not been observed uniformly. In a personal communication, Sanford has noted that several cell strains have shown an inability to return to their former protein medium after being maintained for a few months in a medium composed of chemically defined components and 10 percent horse serum.

Summary and Conclusions

Cell strain NCTC 2071, established by adaptation of cells of NCTC clone 929, mouse fibroblast cells, to chemically defined, protein-free medium NCTC 109, has now been maintained for more than 2 years. The present work was undertaken 1) to study the efficacy of this medium in long-term tissue culture by determining whether other cell lines of clone 929 could be similarly adapted, 2) to study in more detail the adaptation process, and 3) to study the behavior of strain 2071 cells in the readaptation to protein-containing and to chemically defined media.

The adaptation of additional lines of clone 929 to NCTC 109 confirms

the earlier report on the ability of this completely chemically defined, protein-free medium to support these cells as a well-stabilized strain in a state of reasonably rapid proliferation in continuous *in vitro* culture.

The major findings of this study have been:

1) Ten cell lines of clone 929 were successfully adapted to the chemically defined medium in a total of 56 attempts.

2) The cultures in practically all cases underwent either a gradual or abrupt change in cellular morphology, in rate of population growth, and in level of population density.

3) The cultures appeared to go through a critical period which varied widely in regard to time of onset, severity, and duration.

4) Cell lines that successfully adapted to the chemically defined medium ultimately became adequately stable and acquired sufficient growth rates to allow weekly serial 1-to-2 subculturing.

5) Cells of strain 2071 can be readily readapted to horse serum:chick-embryo extract:saline medium and then, subsequently, readily returned to chemically defined medium. Modifications in cell morphology and population-density level accompanied each transfer of the cells to the alternate type medium.

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PLATES

PLATE 79

A sequence of cultures in experiment 2347 showing clone 929 cells passing through an extended "critical period" in the process of successfully adapting to chemically defined, protein-free medium NCTC 109. All figures except those otherwise designated are of representative culture areas. All figures × 200

FIGURE 1.—Second-generation culture at 7 days. Cells in NCTC 109 with 1/4 percent horse-serum supplement for 16 days. *Note* predominance of relatively small, compact cells, which is characteristic of clone 929 cells in proteinic medium.

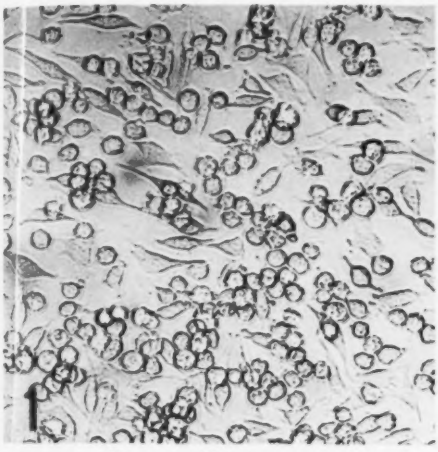
FIGURE 2.—Seventh-generation culture at 1 day. Cells had been in NCTC 109 with 1/4 percent horse-serum supplement for 35 days and 5 generations and, subsequently, in NCTC 109 alone for 14 days and 2 generations. *Note* predominance of large, sheetlike cells with extended processes.

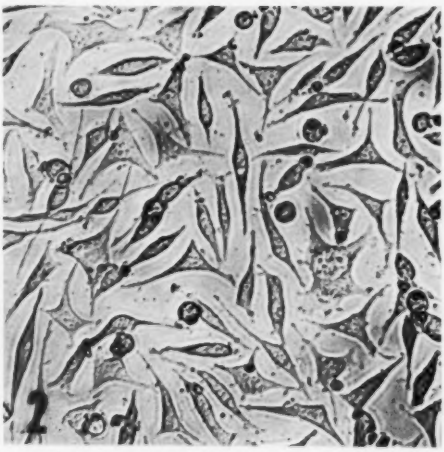
FIGURE 3.—Same culture as in figure 2, showing degenerative condition of cells 34 days later. *Note* sparse population and increased tendency toward bizarre shapes with numerous processes and pronounced granularity.

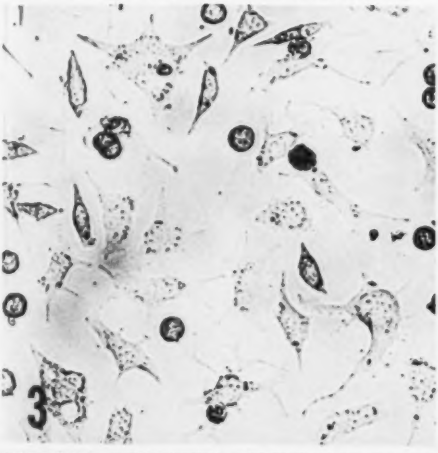
FIGURE 4.—The single culture, at 23 days, through which this cell line ultimately adapted to NCTC 109. Cells in NCTC 109 alone for 4 generations and 91 days. Selected area of culture showing dense cell growth in a single small area from which cell line subsequently adapted and survived. Sister culture did not survive over a week.

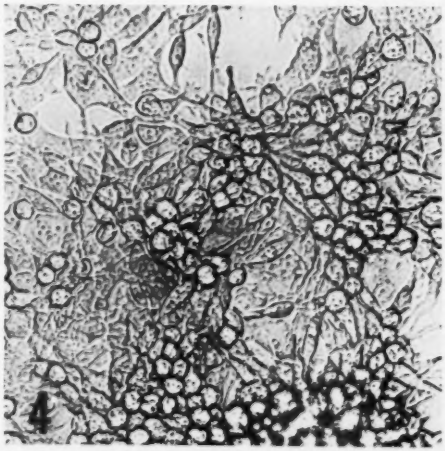
FIGURE 5.—Same culture as in figure 4, at 43 days. Selected area showing dense cell growth in one of the new, discrete areas of growth that arose from cells shaken from the single, small, original area of figure 4. Cell line has been in NCTC 109 for 111 days.

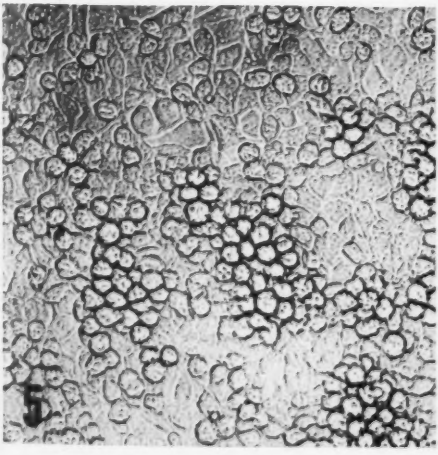
FIGURE 6.—Culture at 6 days of the eleventh generation in NCTC 109. Cell line in NCTC 109 for 166 days. Cells appear to be completely adapted to chemically defined medium with a 1-to-2 culture transfer being made weekly following a 6-week period in which the cell line remained at a low population level in 1 flask.











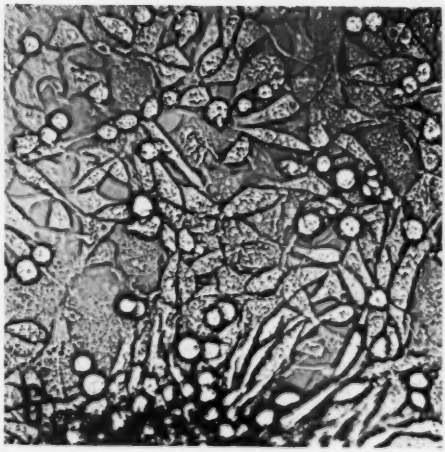


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PLATE 80

A sequence of cultures of experiment 2348 showing the successful adaptation of a line of clone 929 cells to chemically defined, protein-free medium NCTC 109. Contrast with plate 81, which shows an unsuccessful attempt at adaptation by a cell subline that originated from the same first-generation culture as this successful line. All figures are of representative culture areas. All figures × 200

FIGURE 7.—Fourth-generation culture at 14 days. Cells in NCTC 109 for 49 days.

FIGURE 8.—Fifth-generation culture at 5 days. Cells in NCTC 109 for 61 days. *Note* predominance of large, multiprocessed cells as cell line continued in chemically defined medium.

FIGURE 9.—Sixth-generation culture at 6 days. Cells in NCTC 109 for 77 days. *Note* sparse population and continued large, bizarre-shaped cells.

FIGURE 10.—Twelfth-generation culture at 7 days. Cells in NCTC 109 for 126 days; culture appeared to be well adapted to new medium. *Note* increased population density.

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PLATE 81

A sequence of cultures from experiment 2348 showing the failure of a line of clone 929 cells to adapt to NCTC 109. This cell subline originated from the first-generation culture of the successfully adapted cell line shown in plate 80. All figures are of representative culture areas. All figures × 200

FIGURE 11.—Second-generation culture at 8 days. Cells in NCTC 109 for 22 days.

FIGURE 12.—Third-generation culture at 5 days. Cells in NCTC 109 for 27 days. *Note* healthy appearance of culture and graceful, spindle-shaped cells.

FIGURE 13.—Sixth-generation culture at 7 days. Cells in NCTC 109 for 49 days. *Note* sparse population and bizarre, sheetlike, multiprocessed cells.

FIGURE 14.—Same culture as in figure 13, at 35 days. Cells in NCTC 109 for 77 days. *Note* sheetlike appearance of cells and predominance of granularity.

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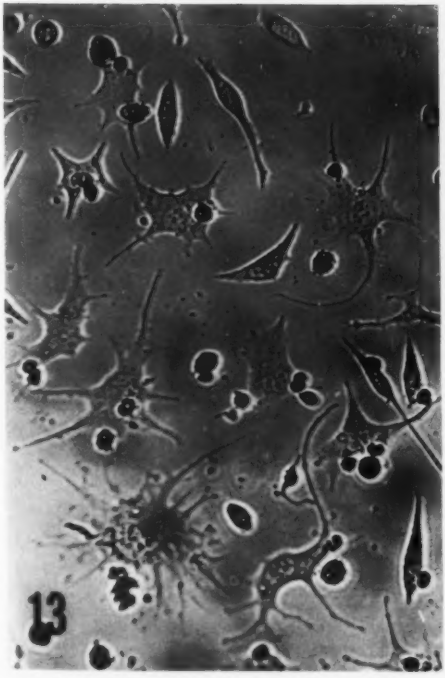
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PLATE 82

Comparison of fibroblast cells in chemically defined medium NCTC 109 with similar cells in horse serum:chick-embryo extract:saline medium at 42 hours in present flasks. All cultures inoculated relatively lightly; photographed areas selected for approximate comparability of cell-population density. Compare with plate 83. All figures × 200

FIGURE 15.—From experiment 2349. Cells in NCTC 109 for 224 days. *Note* predominance of sheetlike cells with numerous processes.

FIGURE 16.—Strain 2071. In NCTC 109 for 22 months. *Note* similarity of cells with those of figure 15.

FIGURE 17.—Parent clone 929 cells still in horse serum:chick-embryo extract:saline medium. *Note* predominance of the more compact, rounded, and spindle-shaped cells.

FIGURE 18.—From experiment 2557. Cells readapted to horse serum:chick-embryo extract:saline medium for 49 days after being in NCTC 109 medium (as strain 2071) for 19½ months. *Note* tendency to resume the more compact shape of cells shown in figure 17.

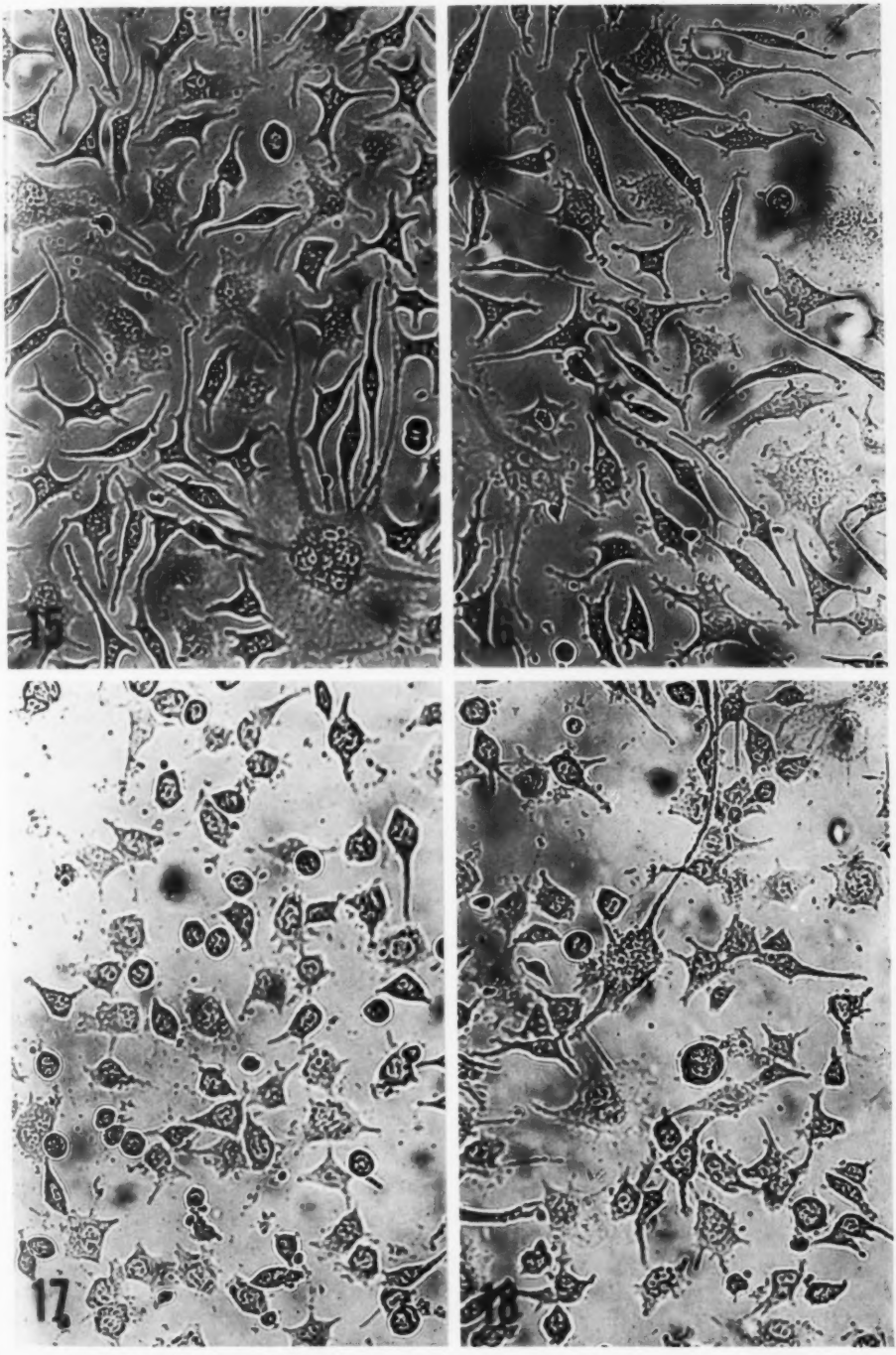


PLATE 83

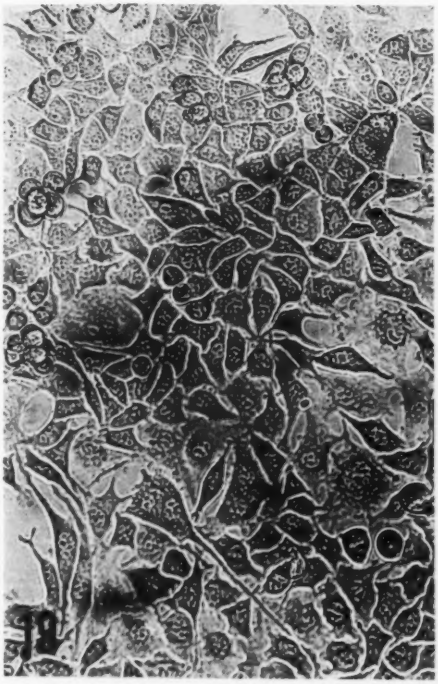
Same cell cultures as shown in plate 82 but at 7 days in culture flasks. The different appearance of cells in chemically defined and in protein media under conditions of cell crowding is shown. All figures × 200

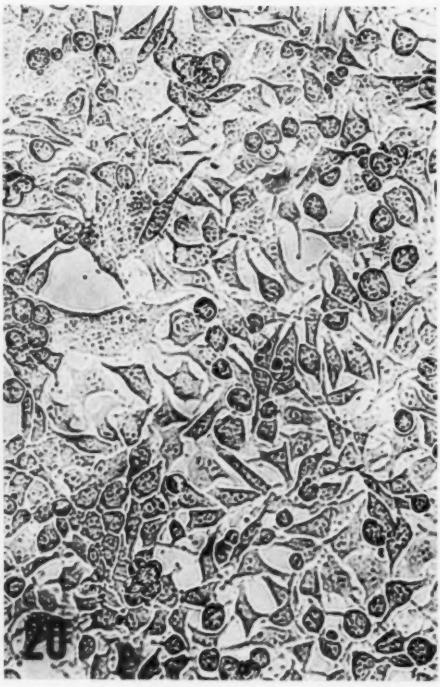
FIGURE 19.—From experiment 2349. Cells in NCTC 109 for 229 days. *Note* tendency of cells to retain sheetlike appearance.

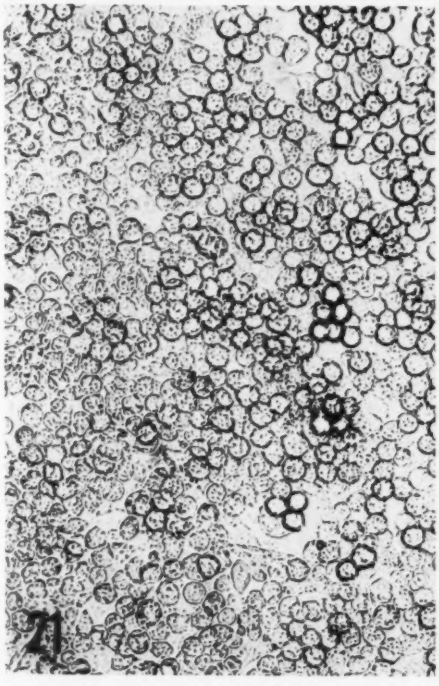
FIGURE 20.—Strain 2071. In NCTC 109 for 22 months.

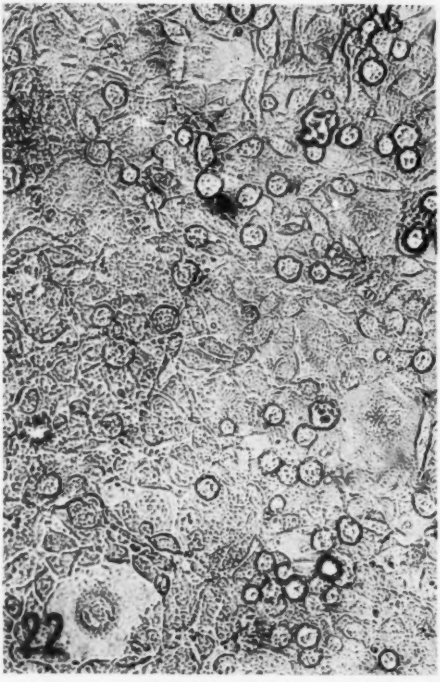
FIGURE 21.—Parent clone 929 cells still in horse serum:chick-embryo extract:saline medium. *Note* greater cell-population density and predominance of small compact cells.

FIGURE 22.—From experiment 2557. Cells readapted to horse serum:chick-embryo extract:saline medium for 54 days after being in NCTC 109 medium (as strain 2071) for 19½ months. *Note* tendency of cells to resume more compact appearance, to appear more cohesive, and to attain a population density more like that shown in figure 21.









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Virus of Avian Erythroblastosis. V. Adenosinetriphosphatase Activity of Blood Plasma from Chickens with the Disease [1,2]

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Myeloblastosis (*1*) and erythroblastosis (*2*) are distinct forms of virus-induced leukemias that occur in the chicken under natural conditions. The former is a highly malignant disease of the myeloid tissues of the hematopoietic system, which is almost always fatal. The latter is a less profound, though analogous and equally fatal, hyperplasia of the erythroid elements. Both are readily transmitted to normal birds of susceptible strains by filtrates of the plasma from diseased hosts (*3*, *4*). The agent responsible for myeloblastosis has been isolated and identified by physical, chemical, and biologic procedures (*5–7*). Identification of the erythroblastosis virus has been limited, so far, to studies by precipitation with chicken antiviral immune serum (*8*).

An outstanding property of the plasmas of many chickens with myeloblastosis is the enzymatic activity to dephosphorylate adenosine- (*5*, *9*) and inosinetriphosphates (*10*). Systematic studies by correlated physical, chemical, and serologic procedures (*5–7*, *11*) have provided substantial evidence that the enzymatic activity is quantitatively related to the virus particle and inseparable from it by the methods employed. The findings in the extensive experiments made possible by access to relatively large amounts of virus were interpreted to indicate that the enzyme was either a constituent of the virus or a material firmly bound to the particle while inside the host cell.

On undertaking investigations for the purpose of the isolation and identification of the virus of erythroblastosis, it was expected that this agent, too, might exhibit a similar enzymatic activity. Examination of numerous plasmas from birds dying of erythroblastosis, which were known to be highly virulent in the transmission of the disease to other birds, failed to reveal activity (*4*) on application of the microscreening adenosinetriphosphatase test (*12*) employed with myeloblastosis. Preliminary experiments with methods of greater sensitivity showed that, although plasmas from birds with erythroblastosis were somewhat more active

[1] Received for publication May 27, 1957.

[2] This work was supported by research grants to Duke University from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; from the American Cancer Society, Inc., on recommendation of the Committee on Growth; and by the Dorothy Beard Research Fund.

than those from normal chickens in the hydrolysis of adenosinetriphosphate, none was comparable with the plasmas from animals with myeloblastosis. It was learned early (*8*) that the physical concentration of erythroblastosis virus particles was much lower in the plasmas of affected birds than in the plasmas containing myeloblastosis virus, but the difference did not seem sufficiently great to account for the exceedingly low enzyme activity of ultracentrifugal concentrates of the agent of erythroblastosis.

Because of the apparent discrepancy, experiments have been made to determine whether or not the erythroblastosis virus displays any adenosinetriphosphatase activity and to make measurements for comparison with the values of the specific activity of the virus of myeloblastosis. These experiments with erythroblastosis virus have been severely limited by the small amounts of virus available; nevertheless, the studies, which have been based on ultracentrifugation and particle counts in relation to adenosinetriphosphatase activity, have sufficed to show that the virus of erythroblastosis possesses little, if any, of the enzyme. The results of the investigations are reported in this paper.

Materials and Methods

Viruses.—The erythroblastosis virus investigated was the same strain (*4*), Engelbreth-Holm's strain "R" (*13*), employed in the earlier studies from this laboratory. No change has been observed in host response to it or in the character of the disease as seen in gross examinations of the birds, or in blood smears made routinely. Comparative studies were made on myeloblastosis virus of the BAI strain A under study here (*14, 15*) for the past 8 years. This virus, too, has retained its initial properties with respect to physical, chemical, and biologic behavior.

Plasmas and virus concentrates.—Blood for erythroblastosis virus was drawn (*4*) into heparin solution by heart puncture from birds near death with the disease. Donors of myeloblastosis virus were selected by the micro-adenosinetriphosphatase test (*12*). Cells were removed by centrifugation, and the plasmas were fresh or were frozen quickly in an ethanol-solid CO_2 bath and stored under crushed CO_2 ice until needed. If the plasmas had been frozen before use, the samples were thawed in a water bath, chilled in ice, and centrifuged again. Those to be inoculated into chickens were then filtered through celite #512 and through Selas filters of 02 or 015 porosity. Some of the plasmas, for measurements of enzyme activity and particle count only, were not filtered. Their behavior in these 2 tests did not differ from that of the filtered material. Studies were made on both plasmas and concentrates of the viruses. Concentration was usually effected by spinning the plasma in an air-driven centrifuge with the rotor turning in an evacuated chamber. In the manipulations described with the different experiments, the preparations were kept at 0 to 5° C. as much as possible.

Measurement of enzyme activity.—Adenosinetriphosphatase activity was determined by measuring the inorganic phosphorus liberated on incubation

of the enzyme material with adenosinetriphosphate. Preliminary experiments showed that the conditions for determining the enzyme activity of myeloblastosis virus (*9*) were suitable for studying the plasmas and virus from chickens with erythroblastosis. The *p*H optimum for activity of both plasma and the fractions obtained by ultracentrifugation was between 7 and 8. It was also observed that, in the presence of 0.04 *M* $MgCl_2$ and 0.05 *M* NaCl, the addition of $CaCl_2$ and KCl had no significant effect. Most of the incubations of enzyme and substrate were carried out at 30° C., for 30 minutes, in a medium containing 0.04 *M* $MgCl_2$, 0.05 *M* NaCl, and 0.05 *M* tris(hydroxymethyl)aminomethane (as the chloride or acetate) at *p*H 7.2. The substrate was the disodium salt of adenosinetriphosphate (crystalline, Sigma Chemical Co., St. Louis, Mo.).

Determinations were carried out on 2 scales:

1) The incubation mixture was of 1 ml. volume and contained 0.6 mg. disodium adenosinetriphosphate as the trihydrate. After incubation, the protein was precipitated with perchloric acid in a final concentration of 0.6 *M* and removed by centrifugation. A volume of 0.8 ml. of the supernatant fluid was taken for inorganic-phosphorus determination by the method of Fiske and Subbarow (*16*). Optical densities were determined in 1 ml. volumes, in the Coleman spectrophotometer model 6A, at a wave length of 700 mμ. Blank tubes contained adenosinetriphosphate without plasma or plasma without adenosinetriphosphate. The procedure, which was suitable for the determination of about 2 to 15 μg. of inorganic phosphorus, required about 100 μl. of a normal plasma or about 0.2 μl. of a strong myeloblastosis plasma.
2) In the second series of determinations, the incubation mixture was 0.075 ml. and contained 15 μg. of disodium adenosinetriphosphate. The incubation mixture also contained 300 μg. of bovine-serum albumin (crystalline, Pentex, Inc., Kankakee, Ill.) as a protective agent. Blank tubes were prepared either, as in procedure 1, with separate tubes for adenosinetriphosphate without enzyme or enzyme without adenosinetriphosphate or, as adopted later, with enzyme (virus or plasma material) heated 5 minutes in a boiling-water bath before addition of substrate. After incubation the tubes were chilled in ice, the protein was precipitated with perchloric acid (0.3 *N*), and a 50 μl. aliquot of the supernatant fluid was used for the determination of inorganic phosphate by the method of Schaffer, Fong, and Kirk (*17*). Optical density was determined in a capillary cell of 2 mm. bore and 50 mm. length in the Beckman DU spectrophotometer at a wavelength of 725 mμ. The range was about 0.01 to 0.25 μg. of phosphorus, which required about 1 μl. of a normal plasma or about 0.003 μl. of a strong myeloblastosis plasma.

The precision of assay varied with the material being tested. In normal and erythroblastosis plasmas, the low level of activity and the relatively large amount of plasma required for testing resulted in large blanks due to the inorganic phosphate of the plasma. The average

deviation of the values of enzyme activity from the mean was about 6 percent for erythroblastosis plasmas with the Fiske-Subbarow procedure. Average deviation, as percent of the mean, was less for myeloblastosis plasmas, and less with the smaller number of determinations done on the microliter scale (about 3%). The precision with supernatant fluids was somewhat poorer, and with pellets was better or worse, depending chiefly on activity.

The adenosinetriphosphatase activity is expressed in terms of μg. of inorganic phosphorus liberated per minute per ml. of plasma or virus suspension, or per 10^{10} particles.

Particle counts were made in the usual way by the procedures involving sedimentation of the virus on agar (*18*). Data of considerable accuracy could be obtained with the plasmas containing high concentrations of myeloblastosis virus—about 10^{12} particles per ml. of plasma. Special problems were introduced by the low concentrations of the erythroblastosis virus in the plasmas. Thus, in the specimens containing about 10^{9} to 10^{11} particles per ml., there was a relatively large proportion of amorphous material that interfered with the counting. For this reason the estimates of particle numbers of erythroblastosis virus were not so good as those of the myeloblastosis agent, but they were still of sufficient accuracy to permit significant interpretations.

Titration of virus infectivity was made by the latent-period procedure (time between inoculation and death of the host) employed with the agent of erythroblastosis (*4*). Inoculations of 0.1 ml. volumes of test preparations were made intravenously into chicks of 14 to 16 days of age (*19*). The host was the line 15 White Leghorns (*20*) developed (*21*) at the Regional Poultry Research Laboratory, East Lansing, Michigan.

Results

Activity of Plasma

It has been mentioned in this and earlier reports (*4, 8*) that plasmas from birds with erythroblastosis did not exhibit sufficient adenosinetriphosphatase activity to show a response in the microscreening test (*12*), nor did particulate material from such plasmas, concentrated manyfold, give the reaction. In the present work, further study by more sensitive methods has shown that the erythroblastosis plasmas exert the enzymatic activity in small degree, though at a somewhat higher level than the plasmas from birds that were apparently normal. The results of such studies with erythroblastosis and normal plasmas are given in table 1 in which the findings with plasmas from chicks with myeloblastosis are included for comparison. Here, it is seen that the normal plasmas were only weakly active, as expected (*22, 23*). The activity of the erythroblastosis plasmas ranged from a comparable level to one approximately tenfold that of the normals. In contrast, the mean activity of the 26 myeloblastosis plasmas was more than 100-fold that seen with

erythroblastosis plasmas. It should be noted that the average activity of the myeloblastosis plasmas was biased somewhat toward high values because the specimens tested included some chosen specially for high virus concentrations. Despite this, however, it is clear that the erythroblastosis plasmas were markedly less active than those occurring in myeloblastosis. Of 39 erythroblastosis plasmas tested, only 3 showed adenosinetriphosphatase activity greater than 3γP per ml. per minute.

TABLE 1.—*Adenosinetriphosphatase activity of plasmas from chickens with erythroblastosis*

Plasma	No. of plasmas	Adenosinetriphosphatase activity (γP/min./ml.)			
		Mean	Range	Standard deviation	Coefficient of variation (*percent*)
Erythroblastosis	39	2.3	0.7–9.6	1.5	65
Myeloblastosis	26	340	44–1060	240	70
Normal	4	0.75	0.42–0.89	0.19	26

Specific Particle Activity

In the foregoing experiments it was demonstrated that the plasmas *per se* from birds with erythroblastosis contained less enzyme than those from chickens with myeloblastosis. A further step in the investigations consisted in studies designed to provide an estimate of the specific activity (activity per particle) in erythroblastosis for comparison with analogous values for the virus of myeloblastosis. For this it was necessary to separate the particles believed to be the virus of erythroblastosis (*8*) from nonviral enzyme present, possibly, in the chicken plasma. Centrifugation seemed to be the mildest effective technique for this purpose, though it was not ideal; for, if some of the nonviral enzyme proved to be particulate, as did appear to be the case, the separation of viral and nonviral adenosinetriphosphatases would be incomplete.

Three types of centrifugation experiments were performed.

A. The results with 4 plasmas are shown in table 2. They were centrifuged once, for 45 minutes, at 15,700 × g in 1.5 ml. volumes in a swinging bucket rotor (*24*). The upper 1.0 ml. of the supernatant fluid was pipetted off, and the pellet was resuspended in the remaining 0.5 ml. of the supernatant fluid. This procedure was employed to avoid damage to the particles that might occur as a result of change in environment, though no such damage has been noted in the resuspension of myeloblastosis virus adenosinetriphosphatase in various media. Enzyme assays and particle counts were made on the fractions. Because the volume was known, it was possible to correct the enzyme activity and particle count of the pellet fraction for the volume of supernatant fluid present and to give a "corrected" pellet value as indicated in table 2.

TABLE 2.—*Distribution of adenosinetriphosphatase activity and particles in ultracentrifugal fractions of plasma from birds with erythroblastosis*

	Fraction	ATPase (γP/ml./min. orig. plasma)	Particles per ml. ($\times 10^{10}$)	ATPase (γP/min./10^{10} particles)	Percent of whole plasma	
					ATPase activity	Particle number
R 107	Plasma	2. 39	5. 4	0. 44	100	100
	Supernatant	1. 16	0. 26	4. 5	49	5
	Pellet	1. 25	3. 9	0. 32	52	72
	Pellet-corrected	0. 67	3. 7	0. 18	28	69
R 108	Plasma	1. 43	2. 4	0. 60	100	100
	Supernatant A*	0. 63	0. 31	2. 0	44	13
	Supernatant B†	0. 30	—	—	21	—
	Pellet	0. 81	2. 7	0. 30	56	111
	Pellet-corrected	0. 51	2. 5	0. 20	35	106
R 109	Plasma	1. 72	8. 0	0. 22	100	100
	Supernatant A*	0. 67	0. 40	1. 7	39	5
	Supernatant B†	0. 26	—	—	15	—
	Pellet	1. 16	11. 7	0. 10	67	146
	Pellet-corrected	0. 83	11. 5	0. 07	48	143
V 950	Plasma	1. 52	2. 4	0. 63	100	100
	Supernatant A*	0. 85	0. 27	3. 1	56	11
	Supernatant B†	0. 57	—	—	35	—
	Pellet	0. 81	2. 1	0. 39	53	89
	Pellet-corrected	0. 38	2. 0	0. 19	25	83
	Mean‡			0. 16		

*Supernatant fluid after a single centrifugation.
†Supernatant fluid freed of particles by a second high-speed centrifugation.
‡Mean enzyme activity of sedimented particles after correction for activity of suspending medium.

Portions of 3 of these plasmas, R108, R109, and V950, were centrifuged at 63,000 × g for 75 minutes in small horizontal cells (*25*), designed to minimize remixing due to convection or stir-back. The upper two-thirds portion of each supernatant fluid was removed and assayed. The resulting values are given as those of the "particle-free" fraction.

From the data collected in table 2, it is seen that a large part, about 44 to 56 percent, of the enzyme activity was removed from the plasma by centrifugation at 15,700 × g for 45 minutes. Most of this could be accounted for in the pellet taken up in a portion of the supernatant fluid. When corrections were made on the pellet volumes, the amounts of enzyme sedimented were found to be from 25 to 48 percent of the initial activity of the plasma. At the same time, the number of particles left in the supernatant fluid varied from 5 to 11 percent of those in the plasma. Recoveries of the particles in the pellets (corrected) were from 69 to 143 percent. When all the particles were removed by spinning at high speed in the small cells, there remained in the supernatant fluid 15 to 35 percent of the enzyme activity which was not sedimented under conditions that resulted in the elimination of all particles resembling those of the

virus. Calculations of the specific activity from measurements of enzyme activity and particle counts on the pellet (corrected) gave the values 0.07 to 0.20 with a mean value of 0.16 γP per minute per 10^{10} particles.

These results indicated clearly that the enzyme activity was distributed in 3 fractions: (*a*) unsedimented material (very small particles or molecular materials not thrown down at 63,000 $\times$ g for 75 minutes); (*b*) particulate material not included in the counts (sedimented at 63,000 $\times$ g but not at 15,700 $\times$ g); and (*c*) particles judged to be the virus and counted as such in the electron micrographs.

B. The purpose of this second group of studies was to determine the specific adenosinetriphosphatase activity of the particles (activity per particle) of erythroblastosis virus measured in large pools of whole plasma and of the particles carried through a single cycle of sedimentation. Five pools of erythroblastosis plasma were used. In table 3, Pool A, there were 125 ml. from 12 chickens; Pool B, 131 ml. from 10; Pool C, 139 ml. from 11; Pool D, 153 ml. from 11; and Pool E, 144 ml. from 13. The donor hosts, which were chickens from experiments described earlier (*8*, *19*), varied from 7 to 77 days of age at the time of inoculation of the virus. A volume of 112 ml. of each pool was spun in the angle rotor in 15 ml. collodion tubes at 30,000 $\times$ g for 1 hour. The supernatant fluid was poured off, and the tubes were wiped with gauze above the pellets. Suspension of the pellets was made in Ca-free Ringer's solution in a volume of 5 ml. for each pool, and all were spun at 2,000 $\times$ g for 15 minutes to remove the gross aggregates. A single plasma, V942, was centrifuged at 30,000 $\times$ g for 15 minutes, and the pellets were taken up and spun at low speed as were those of the concentrates of the pools.

TABLE 3.—*Adenosinetriphosphatase activity of concentrates of erythroblastosis virus sedimented by a single centrifugal cycle, and the proportion (%) of enzyme, particles, and infectious activity of the whole plasma contained in the concentrates*

Preparation	ATPase (γP/*ml./min.*)	Particles per ml. ($\times 10^{10}$)	ATPase (γP/*min./10^{10} particles*)	Percent of plasma attribute		
				ATPase activity	Particle number	Infectivity
Pool A	29	110	0.26	31	295	85
B	17	40	0.44	31	178	40
C	26	105	0.25	39	111	48
D	21	57	0.36	37	435	41
E	19	46	0.41	33	235	41
V942	0.24	0.64	0.37	6	13	
Mean			0.35			

The studies in this series consisted of measurement of particle content, enzyme activity, and infectious capacity on the plasma pools and on the concentrates as described. The results are shown in table 3.

Adenosinetriphosphatase activity of the various concentrates varied slightly less than twofold and the concentration of particles but little more. A fair correlation between the number of particles and enzyme action was seen. Recovery of the adenosinetriphosphatase activity was remarkably uniform, but much more variation was seen in the results of the particle counts. The variation in infectious activity was within the limits of accuracy of the method of titration.

Of much interest was the relative uniformity of the values of enzyme activity per unit number of particles. Despite the difference of the properties of V942 from those of the pools, the specific activity of the particles obtained by only 15 minutes of spinning was essentially the same as the values obtained with the pools.

C. It was seen in the preceding experiments that some of the enzyme activity was associated with materials of a size smaller than that of the virus particles. In order to separate them more completely from the particles, several studies were made in which the particles were sedimented, resuspended in various media, sedimented again, and resuspended in fresh media. In some cases a low-speed centrifugation to remove gross aggregates was added as a final step in the sequence described. Examinations made on the final concentrates yielded the results given in table 4. The mean activity per unit number of particles was 0.081 P per ml. per minute, which was somewhat smaller than that of the particles in plasma or of those sedimented once.

Inorganic Phosphate Liberated

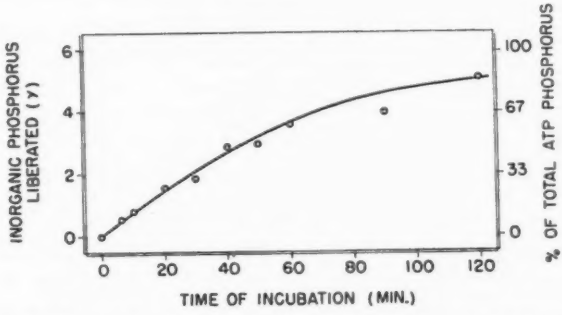
Adenosinetriphosphatase activity of the virus of myeloblastosis was definitely limited to hydrolysis of only the terminal phosphate bond. A difference from this pattern was observed with the plasmas and concentrates of erythroblastosis virus. When a limited amount of substrate was employed, hydrolysis extended beyond the terminal phosphate. This is illustrated by the findings in a typical experiment. For this, 50 μl. of erythroblastosis plasma were incubated for various intervals with 39.2 μg. of adenosinetriphosphate in the 1 ml. assay system. The results are shown in text-figure 1. When a sample of particle concentrate, Pool 2 of table 4, was incubated with 5.9 μg. of adenosinetriphosphate for 30 minutes, 46 percent of the compound was hydrolyzed. If only the terminal phosphate were hydrolyzed, one would expect liberation of only 33 percent of the phosphorus as inorganic phosphate.

Plasma Hemoglobin

It was observed that plasmas obtained from birds near death with erythroblastosis contained much more hemoglobin (determined spectrophotometrically) than was found in plasmas from normal birds or from those with myeloblastosis, though the blood samples were drawn and handled

TABLE 4.—*Adenosinetriphosphatase activity of concentrates of erythroblastosis virus after 2 cycles of sedimentation*

Preparation	ATPase (γP/ml./min.)	Particles per ml. ($\times 10^{10}$)	ATPase (γP/min./10^{10} particles)	Suspending medium
R 68	0. 20	3. 1	0. 063	Ca-free Ringer's solution—0.01 *M* tris acetate, *p*H 7.1
Pool 1	0. 31	2. 5	0. 12	0.25 *M* sucrose
Q 593	0. 33	2. 8	0. 12	0.14 *M* NaCl—0.01 *M* sodium phosphate, *p*H 7.0
Pool 2	5. 2	68	0. 076	0.14 *M* NaCl—0.01 *M* sodium phosphate, *p*H 7.0
Pool P	2. 2	38	0. 057	Ca-free Ringer's solution
Pool O	1. 5	24	0. 062	1.5 percent bovine-serum albumin in Ca-free Ringer's solution
Mean. . .	. . . . .	. . . . .	. 0. 081	



TEXT-FIGURE 1.—Inorganic phosphorus liberated from adenosinetriphosphate by the plasma from a chicken diseased with erythroblastosis.

the same way. This probably indicates the presence in erythroblastosis plasmas of other products of cell breakdown and may account for the slight elevation of adenosinetriphosphatase activity in erythroblastosis plasmas as compared with those from uninfected birds. If some of this enzyme activity is associated with nonviral particulate elements, as it may well be, it would be difficult to separate this increment of enzyme from the erythroblastosis particles by centrifugation.

Discussion

The present observations have shown that the agent of erythroblastosis exhibits little, if any, capacity to dephosphorylate adenosinetriphosphate. Actually, it is doubtful whether the small evidence of activity would have attracted serious attention, even on direct study, had it not been for the

earlier experiments with the myeloblastosis virus (*5–7, 9*). Consideration of all the experiments yields a mean value for specific activity of 0.2γP liberated per minute per 10^{10} particles of erythroblastosis as compared with 2.86γP per minute per 10^{10} particles of myeloblastosis virus. Furthermore, the mean value of 0.08γP per minute per 10^{10} particles for 2-cycle concentrates compared with 0.37 for 1-cycle material indicates that all of even this low activity was probably not intimately associated with the erythroblastosis virus *per se*. Particle preparations were not carried beyond 2 cycles because of the tendency toward aggregation on repeated sedimentation, which made particle counting impractical. It was evident that some of the adenosinetriphosphatase activity of the erythroblastosis plasmas was associated with material that was amorphous in the electron micrographs (compare supernatant fluids A and B of table 2) and with material not responding to the conditions conducive to sedimentation of all the virus particles (supernatant fluids B of table 2). In the present state of experimentation, small case could be made for a specific relationship between the enzyme activity observed and the erythroblastosis virus particles in contrast to the findings with myeloblastosis.

These findings with the erythroblastosis virus are of significance largely, at the moment, because of their bearing on the differentiation of the agent from the virus of myeloblastosis. A major problem of the avian-leukosis complex has been the uncertainty of the etiologic interrelationships (*13*) between the major diseases of this group of neoplasms, namely, lymphomatosis, myeloblastosis, and erythroblastosis. No better illustration of the basis for confusion can be found than by consideration of the complex history of the derivation of the strains of leukemia employed in these studies. That of myeloblastosis was obtained (*26*) initially from birds with lymphomatosis, and, in subsequent passages by Hall, Bean, and Pollard (*26*) and by Johnson (*27*), the manifestations of lymphomatosis, myeloblastosis, and erythroblastosis, and various mixtures of these conditions were observed. It happened, however, that the single bird provided originally by Dr. E. P. Johnson (*14*) must have harbored a pure strain of myeloblastosis (*15*) virus that, thus far, has induced only 1 type of leukemia. It should be mentioned, however, that the schedule of passage has been maintained in a way that did not provide conditions for the detection of lymphomatosis that might have occurred late. Only young chicks, for the most part 3 days of age and, less often, 10 days of age, have been employed. Birds that did not come down with the disease were discarded within 30 to 40 days. One finding has been certain: there has been no evidence of erythroblastosis that involves the red cells or their progenitors in a characteristic way (*2, 4*). Of no less importance was access to the strain of erythroblastosis carried for so long by Engelbreth-Holm (*4, 13*). Derived from a case of myeloid leukosis, it, too, has remained consistent in its behavior in the laboratory at Duke.

The low enzyme activity associated with the erythroblastosis virus constitutes an explicit criterion distinguishing the viruses of the leukemias.

This and the results of other studies have provided the foundation for a major advance in the clarification of the etiologic relationship between the diseases. The status of the problem can be summarized by consideration of (*a*) the differences between the respective viruses and conditions and (*b*) the similarities of the agents.

As noted before, the leukemias differ sharply both in the cell types involved and in the clinical and pathologic manifestations that lead to death of the host (*1, 2, 4, 13*). Host responses to the agents differ in regard to age of the chick at the time of inoculation and to the number of virus particles inducing infection. Young birds are much more susceptible to myeloblastosis (*28*) than older ones, and the opposite is the case in erythroblastosis (*19*). The erythroblastosis virus is approximately 1,000 times more "virulent" than the agent of myeloblastosis in terms of number of particles per infectious dose. Large amounts of virus occur in the plasma in myeloblastosis (*5–7*) and far lesser quantities are seen in erythroblastosis (*8*). Forssman antigen is present in the former, and the agent is neutralized by Forssman antibody (*11*). This phenomenon is not observed with the erythroblastosis virus (*29*). The agents do not behave identically in precipitin (*29*) reactions with specific immune serums from the chicken. Finally, unequivocal differentiation between the 2 agents has been demonstrated by the enzymatic activity of the myeloblastosis virus to dephosphorylate adenosinetriphosphate, a property of dubious association with the virus of erythroblastosis as shown in the present work.

Despite the differences, certain similarities are evident. Morphologically the agents are indistinguishable in electron micrographs (*8, 29*). A close relationship is evident by serologic examination (*29*). Highly effective cross neutralization occurs with the respective immune serums from the chicken. Both agents are neutralized by the serum from rabbits immunized with normal chicken tissue, which yields evidence of the presence in both of antigen behaving as normal host protein. Precipitin reactions are seen with the respective immune serums from the chicken, and the cross-precipitin reaction was observed with antimyeloblastosis chicken immune serum and the erythroblastosis virus. A reverse reaction was not obtained, which might have been due in part, though probably not entirely, to shortcomings of the erythroblastosis immune serum.

From the foregoing it can be concluded that viruses capable of causing myeloblastosis and erythroblastosis can be passed repeatedly in the chicken as separate and distinct filterable agents. It is further evident that the 2 viruses are closely related in their biologic properties as expressed, particularly, by their similarities in antigenic constitution and immunologic behavior. This advance brings to attention more clearly another aspect of the problem, namely, the nature of the relationship concerned with the genesis of the respective agents. At the moment, no direct evidence can be brought to bear on the clarification of this question. In consequence, consideration of the area can be scarcely more than speculation until further data are available. It may be noted, however,

that the leukemias are etiologically related to lymphomatosis (*30*). For this reason and because of the well-known phenomena of derivation of the leukemias from birds with lymphomatosis and the recognized transmissibility of the lymphoid disease in nature (*31*), it is not too remote to consider, for purposes of directing additional investigations, that the virus of lymphomatosis may represent the parent of the leukemia viruses. There is much reason to believe the agents of the leukosis complex are highly labile (*32*) with respect to genetic constitution and the capacity to vary or mutate. Whether the hypothetical progression may be only vertical or horizontal, or both, and reversible as well, is only a matter of conjecture. Nevertheless, the areas for investigation are now more clearly defined, and the results of the studies thus far indicate that both the materials and the methods are available for further clarifications.

Summary

Previous work has shown that the virus of avian myeloblastosis acts strongly to dephosphorylate adenosinetriphosphate. In the present experiments studies were made to determine whether the agent of another avian leukemia, erythroblastosis, exerted a similar activity. Experiments were made with plasma from birds with erythroblastosis and with fractions of the plasma obtained by ultracentrifugation. Examination showed that the adenosinetriphosphatase activity of the erythroblastosis plasmas was slightly greater than that of plasma from normal birds. Studies of the various fractions indicated that a large proportion, about half, of the activity was carried by the plasma constituents too small for sedimentation in centrifugal fields, which eliminated the virus and other particles visible by electron micrography. A second and smaller amount was found with particles of a size range smaller than that of the virus. The remainder was present in the fraction containing the virus particles. The specific adenosinetriphosphatase activity of the erythroblastosis virus, calculated on the basis of direct particle counts, was such that an average of 0.08γP was liberated per minute per 10^{10} virus particles sedimented in 2 cycles of ultracentrifugal fractionation. Comparison of this value with the analogous activity of the myeloblastosis virus, namely, 2.86γP per 10^{10} particles per minute, revealed the relatively inconsequential activity of the erythroblastosis virus. The findings yielded no evidence that adenosinetriphosphatase activity was unequivocally associated with the erythroblastosis virus.

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Virus of Avian Erythroblastosis. VI. Further Studies on Neutralization by Antiserum to Normal Chicken Protein [1,2]

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Earlier reports have described the results of studies on the serologic properties of the viruses of the avian leukemias, myeloblastosis (*1*), and erythroblastosis (*2*). It has been observed that the formalin-inactivated agents induce antibodies in the chicken which are active in the neutralization of infectious capacity and precipitation of the respective viruses. Cross-serologic tests (2) with the chicken serums have provided evidence both of close antigenic kinship and of differences between these leukemia viruses, which, together with much other information, clearly indicates that the two are separate biologic entities.

During the initial studies on the virus of myeloblastosis (*1*), it was found that concentrates of the agent produced immune bodies in the rabbit that not only neutralized infectivity but also fixed complement strongly with such preparations. The precipitation reaction was not observed with these rabbit immune serums. A wholly unexpected phenomenon was found when it was discovered that antibodies from rabbits immunized with normal adult chicken tissue or normal chicken serum likewise neutralized virus infectivity and bound the complement with virus concentrates. Absorption of the rabbit serums with liver and spleen tissue removed most of the neutralizing and complement-binding activities. An additional finding was the neutralization of the agent of myeloblastosis with anti-Forssman antibodies from rabbits injected with guinea-pig kidney, an effect eliminated entirely by absorption of the rabbit serum with sheep red blood cells. In these findings, the neutralizing activity was independent of the complement. The results of these experiments, augmented by correlated physical and chemical analyses (*3–5*), were interpreted to indicate that the myeloblastosis virus contained intrinsic components that were like normal chicken protein and Forssman antigen.

Analogous, though less extensive, experiments have shown that the virus of erythroblastosis (*2*) is neutralized by the same antinormal chicken protein rabbit immune serums that were active against the myeloblastosis

[1] Received for publication May 27, 1957.

[2] This work was supported by research grants to Duke University from the American Cancer Society, Inc., on recommendation of the Committee on Growth; from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; and by the Dorothy Beard Research Fund.

agent. Thus, the interpretations of the data derived from studies of the virus of myeloblastosis, with respect to normal chicken antigen, were applicable to that of erythroblastosis. A difference between the leukemia viruses, however, was the lack of neutralizing activity of Forssman antibodies on the erythroblastosis virus.

Another tumor virus affected by antinormal chicken-tissue immune serums from heterologous hosts is that responsible for the Rous sarcoma (*6*). These serums have been observed by several investigators to suppress infectivity of the virus. This activity, however, occurs only when the virus-serum mixtures contain complement. The phenomenon has been the object of many studies to clarify the mechanism of apparent neutralization, which differs from that occurring with other viruses and their specific antiserums in the requirement of complement. The findings in this field have been discussed recently by Rubin (*6*) who also has reinvestigated the problem. With the chorio-allantoic membrane of the chick embryo, this investigator obtained results which he interpreted to indicate that the action of the serum was not on the virus directly, but on the host cell involved.

Inasmuch as the results of Rubin's experiments might be regarded as having a bearing on the interpretation of the findings with the myeloblastosis and erythroblastosis viruses, as the author implied with respect to the former, it has seemed desirable to make similar studies with one of these agents of avian leukemia. This has been done with the erythroblastosis virus in experiments designed to duplicate, within the limits of the host-virus system employed, those carried out by Rubin with the agent of the Rous sarcoma. The work with the erythroblastosis virus, which is described in the present paper, yielded results entirely unlike those observed by Rubin, and served to substantiate more firmly the evidence that the action of the antinormal host protein immune serum is directly on the agent and independent of the intervention of complement.

Materials and Methods

Virus.—The virus employed was Engelbreth-Holm's strain "R" erythroblastosis (*7*). It was obtained, in February, 1955, from Dr. Astrid Fagraeus (State Bacteriological Laboratory, Stockholm) as described earlier (*8*), and has since been passed only by means of the virus in filtered plasma from diseased birds. The pattern of the response of the host—White Leghorns of the inbred line 15 developed (*9*) at the Regional Poultry Research Laboratory, East Lansing, Michigan—has not changed from that initially reported (*8*).

All the present experiments were made with the agent in a pool "H" of plasma derived from 38 chickens that were 16 days of age at the time of inoculation with erythroblastosis virus. Blood was taken by heart puncture into a heparin solution, and the cells were removed by 2 cycles of centrifugation in the cold room. The plasmas were then pooled in a volume of 115 ml., and centrifuged again at 3,000 $\times$ g for 20 minutes. The pool was parceled in 5 ml. volumes in 20 ml. small-mouth serum vials

that were closed with rubber-sleeve stoppers. After quick freezing in a dry ice-alcohol bath, the material was stored under crushed CO_2 ice. For an experiment, the plasma was thawed in tap water and spun again at 3,000 $\times$ g for 10 minutes. Dilution was made with an equal volume of calcium-free Ringer's solution, and filtration was effected, as usual, with celite and Selas #02 filters (*2*, *3*, *5*). Subsequent dilutions were made with bovine-serum albumin in Simms' solution (BSA-Simms' solution) (*10*).

Immune serums.—Studies were made with 3 serums, which were described in earlier reports (*2*, *11*): 1) antierythroblastosis immune serum prepared by injection of chickens with the agent inactivated with formalin; 2) a pool of serum (CS 1–4) from rabbits immunized by repeated injections of normal chicken serum; 3) a pool of serum (CT 1–4) from rabbits immunized with ground liver and spleen from normal chickens. There was no gross evidence of disease in the birds, which were White Leghorns of line 15, presumed to be "normal." Serum from the pools, stored in small lots at −15° C., was thawed and centrifuged to remove insoluble material. Antierythroblastosis chicken immune serum was heated at 56° C. for 30 minutes immediately before the experiment. The rabbit serums were treated in a like manner and then absorbed 4 times with sheep red blood cells (*1*, *2*). Need for absorption with the red cells was questionable because the agent of erythroblastosis, in contrast to the myeloblastosis virus, is not influenced (*2*) by Forssman antibodies.

Preparation of inoculums.—Although the sequence of manipulations varied with the different experiments, the basic pattern was that routinely employed in neutralization experiments with the avian leukemia viruses. The control virus preparations consisted of 2 or more dilutions of the filtered virus made with BSA-Simms' solution. The notations in the respective tables indicate the volumes of plasma in the 0.1 ml. inoculums introduced into the chickens. Ordinarily, the serum-virus preparations were made by mixing 1.0 ml. of the appropriate dilution of plasma with 1.0 ml. of the desired dilution of serum and diluting the whole to 5.0 ml. in Wassermann tubes by adding 3.0 ml. of BSA-Simms' solution. All the preparations, including the virus standards, were then incubated for 2 hours in the closed tubes at 37° C. with the water of the warm bath well above the fluid levels of the preparations. Afterward, the materials were cooled, and the injections were made as soon as possible. Variations from this pattern are indicated in relation to the respective experiments.

Inoculation.—The test chickens were White Leghorns of line 15, 14 to 17 days of age at the time of inoculation (*12*). Injections were made intravenously in volumes of 0.1 ml. into 30 birds per dose group. Blood smears were made as usual (*2*) for following the course of the disease.

Analysis of results.—Measurement of host response was made on the basis of the latent period, which was the time between inoculation and death of the injected chicken (*2*). The number of chickens dying was noted twice daily, at 9 a.m. and 9 p.m., which yielded data more susceptible to analysis than those based on the 24-hour period initially em-

ployed (*2*) with the erythroblastosis virus. A large proportion of the chickens was grouped close to the median level of susceptibility, and many of the birds died within the 24-hour interval most significant for analysis. The procedures for analysis and for estimation of virus activity and residual infectivity were the same as those previously described (*2*).

Experiments and Results

Experiment 1

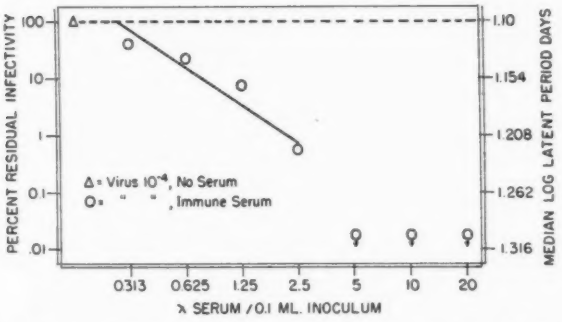
This study describes the neutralizing effect on the erythroblastosis virus of immune serum from the rabbit against normal chicken serum. The virus controls, table 1, were 3 *dilutions* of plasma, $10^{-1.301}$, $10^{-2.301}$, $10^{-3.301}$ which yielded, in 0.1 ml. inoculums, 5.0, 0.5, and 0.05 λ *volumes* of plasma in the chickens. The virus-serum mixtures were prepared with 1.0 ml. of plasma dilution $10^{-2.301}$; 1 ml. of whole or diluted serum; and 3.0 ml. BSA-Simms' solution. The resulting *dilution* of the plasma in the 5.0 ml. volumes was thus 10^{-3}, and the *volume* reaching the chicken was 10^{-4} ml. or 0.1 λ plasma (table 1). These relations were selected to afford a system of sensitivity adequate for detection and measurement of the effects of the immune serum from the rabbit.

TABLE 1.—*Neutralization of virus of erythroblastosis in filtered plasma (Pool H) with the serum (CS 1–4) from rabbits immunized with* ***normal chicken serum*** (see *text-fig. 1*)

Serum	Virus plasma* (λ)	Serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity (*percent*)‡
None	5.0	—	30	29	1.012	—
	0.5	—	30	29	1.074	—
	0.05	—	30	28	1.099	—
Anti-chicken	0.1	20.	30	3	>1.301	<0.04
	0.1	10.	30	3	>1.301	<0.04
	0.1	5.	30	9	>1.301	<0.04
	0.1	2.5	30	16	1.220	0.6
	0.1	1.25	30	28	1.160	7.7
	0.1	0.625	30	29	1.135	23.
	0.1	0.313	29	28	1.119	45.

*Volume per 0.1 ml. inoculum.
†Corrected number of chicks inoculated [*see* (*8*) for method of calculation].
‡Calculated on the basis of latent-period relations.

The results observed in this experiment, table 1, text-figure 1, were like those seen in an earlier experiment [*see* table 3 and text-fig. 3 of (*2*)] which showed the neutralizing effect of antinormal *chicken serum*-rabbit immune serum on the agent of erythroblastosis. The findings with anti-normal chicken-*tissue*-rabbit immune serum [*see* table 2, text-fig. 2 of (*2*)] were like those seen in this experiment. It should be emphasized that the neutralization occurred on incubation of the serum-virus mixtures in the *absence of complement.*



TEXT-FIGURE 1.—Neutralization of the virus of erythroblastosis in filtered plasma (Pool H) with the serum (CS 1–4) from rabbits immunized with *normal chicken serum* (table 1).

Experiment 2

In his experiments with the Rous sarcoma virus, Rubin (*6*) found that when rabbit antiserum was added to normal chick tissue and incubated with the agent and complement, the number of lesions caused by the mixture was far less than that resulting from the same amount of untreated agent. This evidence of apparent neutralization was in line with the findings of the earlier investigators who had made their tests by injection of these incubated serum-complement-virus mixtures into the tissues of the intact host. An extension of the study then showed that the antinormal chick tissue-rabbit immune bodies introduced with complement onto the chorio-allantoic membrane *after* inoculation of the virus was effective to the same extent in lessening the number of lesions due to the agent.

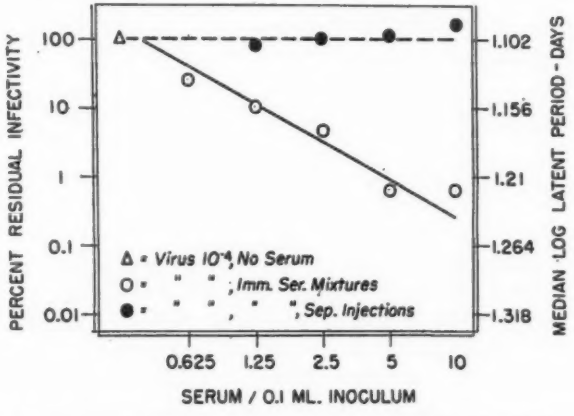
It was of interest now to investigate the effect of antinormal chicken serum-rabbit immune serum on the erythroblastosis virus when the antibody was introduced into the chicken after injection of the agent. For this study the usual series of controls of virus without serum and of virus-serum mixtures were prepared, as shown in table 2. At the same time there were set up: (*a*) 4 tubes containing 1.0 ml. of virus and 4.0 ml. BSA-Simms' solution in which the concentration of virus was the same as that in the serum-virus mixtures; and (*b*) 4 tubes with 1.0 ml. of successive twofold dilutions of serum and 4.0 ml. of BSA–Simms' solution. All the tubes were incubated at 37° C. for 2 hours.

The virus controls, the regular serum-virus mixtures, and the virus preparations containing no serum were inoculated immediately in the usual way. After 3 hours, the serum preparations without virus were injected into the opposite wing vein of the individuals of the respective dose groups that had received the virus without serum. The data of the experiment are given in table 2 and summarized graphically in text-figure 2. It is seen that the virus in the regular serum-virus mixtures was neutralized in the usual pattern, but the serum administered 3 hours later was entirely without effect.

TABLE 2.—*Influence on virus of erythroblastosis in filtered plasma (Pool H) of serum (CS 1–4) from rabbits immunized with* ***normal chicken serum*** (see *text-fig. 2*) *when serum and virus were incubated together and when serum was injected separately* ***3 hours after*** *virus inoculation*

Serum	Virus plasma* (λ)	Serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity (*percent*)‡
None	5. 0	—	30	28	1. 012	—
	0. 5	—	30	28	1. 067	—
	0. 05	—	30	28	1. 115	—
Anti-chicken serum (*regular mixtures*)	0. 1	10	30	9	1. 215	0. 12
	0. 1	5	30	10	1. 215	0. 12
	0. 1	2. 5	30	19	1. 177	4
	0. 1	1. 25	30	23	1. 152	12
	0. 1	0. 625	30	27	1. 132	27
Anti-chicken serum (*serum 3 hrs. after virus*)	0. 1	10	30	28	1. 100	109
	0. 1	5	29	27	1. 090	166
	0. 1	2. 5	30	29	1. 102	100
	0. 1	1. 25	30	26	1. 107	81

*Volume per 0.1 ml. inoculum.
†Corrected number of chicks inoculated [*see* (3) for method of calculating].
‡Calculated on the basis of latent-period relations.



TEXT-FIGURE 2.—Influence on the virus of erythroblastosis in filtered plasma (Pool H) of the serum (CS 1–4) from rabbits immunized with *normal chicken serum* (table 2). ○—Data obtained when virus and serum were incubated together before injection; ●—lack of effect of *the same* volumes of serum injected *3 hours after* inoculation of virus.

Experiment 3

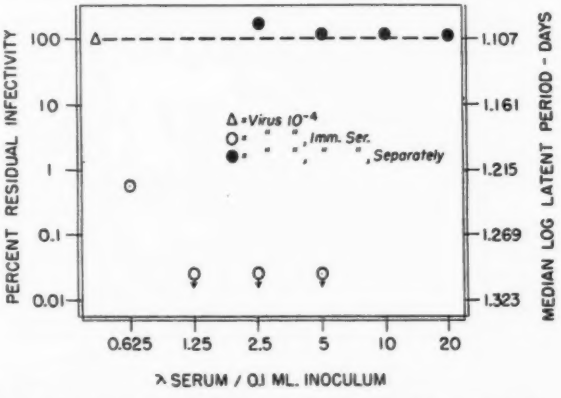
This study was like the preceding one except that the rabbit antibodies were induced by the administration of normal chicken tissue instead of normal chicken serum. In this case the immune-serum injections, in equal and larger volumes (table 3) followed those of the virus preparations without serum, by an interval of 4 hours. Again, it is seen (table 3, text-fig. 3) that no influence was exerted by serum when it was ad-

ministered separately after the virus had been inoculated. Although in equal amounts, it neutralized more than 99 percent of the virus in the regular mixtures.

TABLE 3.—*Influence on virus of erythroblastosis in filtered plasma (Pool H) of serum from rabbits immunized with* ***normal chicken tissue*** (see *text-fig. 3*) *when serum and virus were* ***incubated together*** *and when serum was injected separately* ***4 hours after*** *virus inoculation*

Serum	Virus plasma* (λ)	Serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity (*percent*)‡
None	0. 5	—	30	29	1. 070	—
	0. 05	—	30	29	1. 124	—
Anti-chicken tissue (*regular mixtures*)	0. 1	5	30	4	>1. 301	<0. 26
	0. 1	2. 5	30	5	>1. 301	<0. 26
	0. 1	1. 25	30	6	>1. 301	<0. 26
	0. 1	0. 625	30	21	1. 227	0. 6
Anti-chicken tissue (*serum 4 hrs. after virus*)	0. 1	20	29	27	1. 110	111
	0. 1	10	30	25	1. 112	121
	0. 1	5	30	30	1. 112	121
	0. 1	2. 5	30	27	1. 095	170

*Volume per 0.1 ml. inoculum.
†Corrected number of chicks inoculated [*see* (8) for method of calculation].
‡Calculated on the basis of latent-period relations.



TEXT-FIGURE 3.—Influence on the virus of erythroblastosis in filtered plasma (Pool H) of the serum from rabbits immunized with *normal chicken tissue* (table 3). ○—Data obtained when virus and serum were incubated together before injection; ●—lack of effect of *the same and greater* volumes of serum injected *4 hours after* inoculation of virus.

Experiment 4

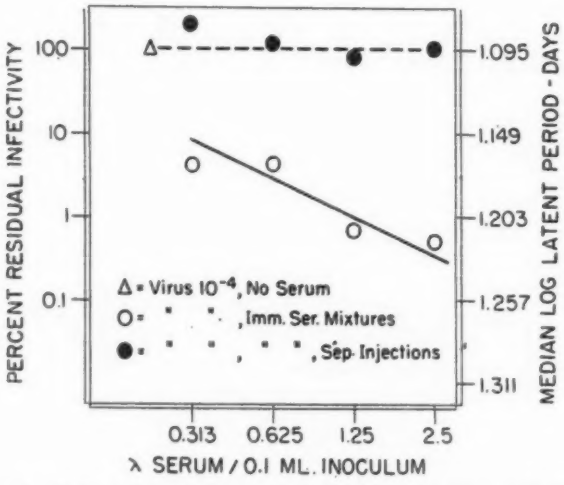
The foregoing experiments indicated clearly that the antinormal chicken protein immune serums from the rabbit produced no demonstrable neutralization if virus and serum were given separately, with the serum following the agent. It was of interest, now, to see whether

an influence would be exerted by serum introduced before the virus. Virus controls, regular serum-virus mixtures, and virus and serum preparations were made as before. The controls and the serum-virus mixtures were inoculated as usual. The serum and virus preparations were then given separately to the individuals of the respective dose groups shown in table 4. A given dose of serum was injected into 1 wing vein of the

TABLE 4.—*Influence on virus of erythroblastosis in filtered plasma (Pool H) of the serum (CT 1–4) from rabbits immunized with* ***normal chicken tissue*** (see *text-fig. 4*) *when virus and serum were* incubated ***together*** *and when serum was injected separately* ***immediately*** before *virus inoculation*

Serum	Virus plasma* (λ)	Serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity (*percent*)‡
None	0. 5	—	27	26	1. 057	—
	0. 05	—	30	26	1. 113	—
Anti-chicken tissue (*regular mixtures*)	0. 1	2. 5	30	10	1. 220	0. 5
	0. 1	1. 25	30	15	1. 212	0. 7
	0. 1	0. 625	30	24	1. 170	4
	0. 1	0. 313	30	25	1. 170	4
Anti-chicken tissue (*serum injected before virus*)	0. 1	2. 5	30	29	1. 095	100
	0. 1	1. 25	30	28	1. 10	81
	0. 1	0. 625	30	28	1. 09	112
	0. 1	0. 313	30	30	1. 077	215

*Volume per 0.1 ml. inoculum.
†Corrected number of chicks inoculated [*see* (*8*) for method of calculation].
‡Calculated on the basis of latent-period relations.



TEXT-FIGURE 4.—Influence on the virus of erythroblastosis in filtered plasma (Pool H) of the serum (CT 1–4) from rabbits immunized with normal chicken tissue (table 4). ○—Data obtained when virus and serum were incubated together before injection; ●—lack of effect of *the same volumes* of serum injected *immediately* before inoculation of virus.

individual, and the virus was inoculated quickly into the vessel of the opposite wing. Only a few seconds intervened between the injections.

The results are illustrated in table 4 and text-figure 4. Again, as before, the virus was neutralized in the mixtures of serum and virus incubated together, but the serum produced no effect on the virus injected immediately afterward.

Experiment 5

With the evidence of lack of effect of antinormal chicken protein rabbit immune serum on virus introduced separately into the chicken, whether the serum or virus were injected first, a test was made to learn whether a similar behavior would be exhibited by antierythroblastosis virus immune serum from the *chicken*. Table 5 and text-figure 5 show the results when the serum was given separately 3 hours after the virus. No suppression of virus activity occurred under these conditions.

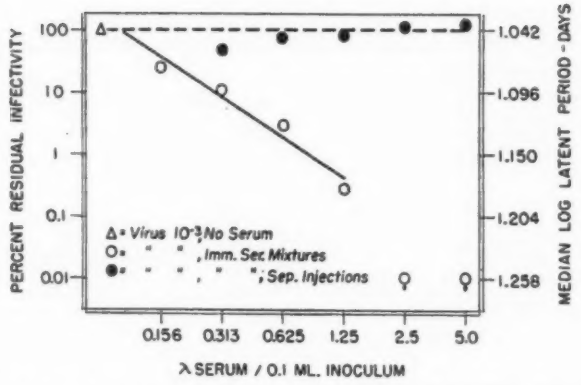
TABLE 5.—*Influence on the virus of erythroblastosis in filtered plasma (Pool H) of the serum of chickens immunized with formalin-inactivated concentrate of virus of erythroblastosis (see text-fig. 5) when serum and virus were* ***incubated together*** *and when serum was injected separately* ***3 hours after*** *virus inoculation*

Serum	Virus plasma* (λ)	Serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity (*percent*) ‡
None	5.0	—	30	29	1.020	—
	0.5	—	30	30	1.061	—
	0.05	—	30	30	1.095	—
Antiviral (*regular mixtures*)	1	5	30	1	>1.301	<0.01
	"	2.5	30	5	>1.301	<0.01
	"	1.25	30	19	1.180	0.28
	"	0.625	30	28	1.125	2.9
	"	0.313	30	30	1.095	10
	"	0.156	30	30	1.075	24
Antiviral (*serum 3 hrs. after virus*)	1	5	30	29	1.037	123
	"	2.5	30	28	1.040	109
	"	1.25	29	29	1.047	81
	"	0.625	30	29	1.049	74
	"	0.313	29	29	1.060	46

*Volume per 0.1 ml. inoculum.
†Corrected number of chicks inoculated [*see* (5) for method of calculation].
‡Calculated on the basis of latent-period relations.

Experiment 6

Another type of experiment made by Rubin with the Rous sarcoma virus was concerned with an apparent reversal of the effect of serum on the virus by diluting the mixtures *after incubation*. This experiment and the following one were made to test the possible occurrence of a like phenomenon with the virus of erythroblastosis. In addition to the virus controls and the regular serum-virus mixtures, special preparations were made of serum and virus as shown in table 6. To 4 successive twofold



TEXT-FIGURE 5.—Influence on the virus of erythroblastosis in filtered plasma (Pool H) of the serum of chickens immunized with formalin-inactivated concentrate of the virus of erythroblastosis (table 5). ○—Data obtained when virus and serum were incubated together before injection; ●—lack of effect of *the same volumes* of serum injected *3 hours after* inoculation of the virus.

dilutions of virus in 2.0 ml. volumes, as indicated, were added 2.0 ml. of a 1:4 dilution (1 part whole serum to 3 parts BSA-Simms' solution) of antinormal chicken tissue-rabbit immune serum. All the preparations were incubated for 2 hours at 37° C. The special mixtures were then diluted appropriately with BSA-Simms' solution to bring the relative concentrations of serum and virus to the same levels as those obtained in the regular mixtures.

The results of the present experiment are given in table 7 and text-figure 6. The findings with the regular mixtures were the same as usual. It is evident that the effect of the serum on virus in the mixtures diluted after incubation was much greater than that of the same amounts of serum

TABLE 6.—*Protocol of experiment of table 7 and text-figure 6* (see *text*)

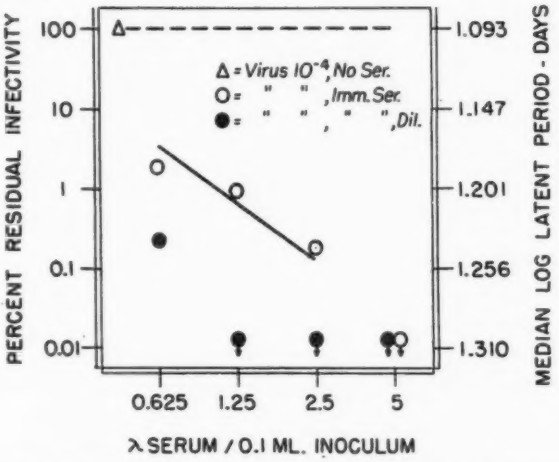
Serum	Virus plasma		Serum		Diluent*		Mixture	Diluent*
	Dil.	Vol. (*ml.*)	Dil.	Vol. (*ml.*)	Vol. (*ml.*)		Vol. (*ml.*)	Vol. (*ml.*)
None	$10^{-2.3}$	—	—	—	—	Incubated 2 hrs. at 37° C.	—	—
	$10^{-3.3}$	—	—	—	—		—	—
Anti-chicken tissue (*regular mixtures*)	$10^{-2.3}$	1.0	1:4	1.0	3.0		—	—
	"	"	1:8	"	"		—	—
	"	"	1:16	"	"		—	—
Anti-chicken tissue (*mixtures diluted after incubation*)	$10^{-1.4}$	2.0	1:4	2.0	—		1.0	19.0
	$10^{-1.7}$	"	"	"	—		"	9.0
	$10^{-2.0}$	"	"	"	—		2.0	8.0
	$10^{-2.3}$	"	"	"	—		"	3.0

*Bovine-serum albumin in Simms' solution (*see* text).

TABLE 7.—*Influence on virus of erythroblastosis in filtered plasma (Pool H) of the serum (CT 1–4) from rabbits immunized with* ***normal chicken tissue*** (see *text-fig. 6*) *when serum and virus were* ***incubated together*** *in the usual way and when final dilutions of incubated mixtures of serum and virus were made* ***after incubation*** (see *table 6*)

Serum	Virus plasma* (λ)	Serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity (*percent*)‡
None	0. 5	—	30	28	1. 060	—
	0. 05	—	30	27	1. 107	—
Anti-chicken tissue (*regular mixtures*)	0. 1	5. 0	30	3	>1. 301	<0. 1
	0. 1	2. 5	30	10	1. 242	0. 2
	0. 1	1. 25	30	18	1. 202	0. 9
	0. 1	0. 625	30	19	1. 187	2
Anti-chicken tissue (*mixtures diluted after incubation*)	0. 1	0. 625	30	8	1. 233	0. 3
	0. 1	1. 25	30	3	>1. 301	<0. 1
	0. 1	2. 5	30	5	>1. 301	<0. 1
	0. 1	5. 0	30	3	>1. 301	<0. 1

*Volume per 0.1 ml. inoculum.
†Corrected number of chicks inoculated [*see* (*5*) for method of calculation].
‡Calculated on the basis of latent-period relations.



TEXT-FIGURE 6.—Influence on the virus of erythroblastosis in filtered plasma (Pool H) of the serum (CT 1–4) from rabbits immunized with *normal chicken tissue* (table 7). ○—Data obtained when virus and serum were incubated together before injection; ●—results observed with mixtures of the *same amounts* of serum and virus diluted (table 6) *after* incubation.

in the regular mixtures. A better illustration is provided by the next experiment.

Experiment 7

This represents an extension of the preceeding study, and was made in the same way, but with a sufficient number of preparations to reveal the

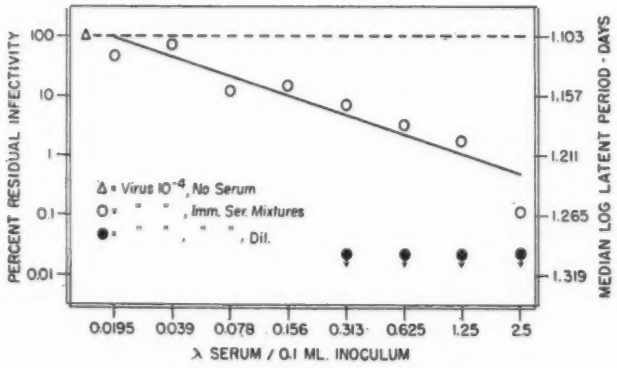
happenings more completely. In this instance, table 8, text-figure 7, it is evident that complete reversal of the neutralizing effect did not occur on dilution of the special serum-virus mixtures after incubation in the concentrated preparations. It is clear from the conditions of the experiments that *some* reversal could have occurred beyond the limits of detection by the method of study. Nevertheless, if any reversal took place, it could not be observed and was certainly not complete as interpreted by Rubin from the results of his investigations with the Rous sarcoma virus.

Host weights and blood volumes.—The conditions under which the inoculations into chickens were made differed radically from those prevailing

TABLE 8.—*Experiment like that in table 6, except for use of different amounts of immune serum* (see *text-fig. 7*)

Serum	Virus plasma* (λ)	Serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity (*percent*)‡
None	0. 5	—	30	28	1. 065	—
	0. 05	—	30	29	1. 124	—
Anti-chicken tissue (*regular mixtures*)	0. 1	2. 5	30	11	1. 262	0. 1
	0. 1	1. 25	30	18	1. 197	2
	0. 1	0. 625	30	25	1. 183	3
	0. 1	0. 313	30	27	1. 165	7
	0. 1	0. 156	30	26	1. 141	19
	0. 1	0. 078	30	29	1. 152	12
	0. 1	0. 039	30	29	1. 110	73
	0. 1	0. 0195	30	30	1. 12	47
Anti-chicken tissue (*mixtures diluted after incubation*)	0. 1	0. 313	30	6	>1. 301	<0. 02
	0. 1	0. 625	30	3	>1. 301	<0. 02
	0. 1	1. 25	29	6	>1. 301	<0. 02
	0. 1	2. 5	29	4	>1. 301	<0. 02

*Volume per 0.1 ml. inoculum.
†Corrected number of chicks inoculated [*see* (*8*) for method of calculation].
‡Calculated on the basis of latent-period relations.



TEXT-FIGURE 7.—This experiment was like that of text-figure 6 except for the use of different amounts of immune serum (table 8).

in the administration of materials onto the chorio-allantoic membrane of the chick embryo. Under the latter circumstances, as in the studies on the agent of the Rous sarcoma (*6*), virus-serum mixtures, virus alone, or serum alone were placed directly on the host cells to be affected. There was essentially no dilution, and the total amount of reactant was in immediate contact with the cells. In the work with erythroblastosis, the fluids were introduced in 0.1 ml. volumes into the circulation, whereupon dilution occurred commensurate, initially, with the volume of the plasma. Subsequent dilution probably occurred with escape of material from the blood stream, possibly, before all could be brought into approximation with susceptible cells.

An estimate of the order of the resulting dilution can be made by calculations of the approximate blood volumes of the injected chickens. This was accomplished by weighing the various dose groups of several experiments and obtaining the mean weights of the individual chickens for each dose group. The results for experiments 2 and 5 are given in table 9. Assuming a blood volume of 1/13 of the body weight, the mean volume per chicken in experiment 2 was 11.4 ml. and for experiment 5, 11.3 ml. Since dilution was related only to the plasma, which was not less than half the whole blood volume, it can be judged that the order of dilution was about 50-fold. A factor of this magnitude provides a ready explanation for the failure of immune serum to influence virus activity when the 2 were administered separately. Consideration of such dilution emphasizes the firmness of the bond established between the immune serum and virus during the incubation of mixtures before inoculation.

TABLE 9.—*Mean weights and blood volumes of chickens in the various dose groups of experiments 2 and 5*

Dose group*	EXPT. 2		EXPT. 5	
	Weight (*gm.*)	Blood volume† (*ml.*)	Weight (*gm.*)	Blood volume† (*ml.*)
1	153. 4	11. 8	142. 0	10. 9
2	151. 4	11. 7	143. 8	11. 1
3	152. 2	11. 7	147. 7	11. 4
4	151. 4	11. 7	150. 5	11. 6
5	151. 4	11. 7	143. 7	11. 1
6	147. 7	11. 4	147. 7	11. 4
7	149. 6	11. 5	140. 0	10. 8
8	142. 9	11. 0	147. 7	11. 4
9	145. 8	11. 2	156. 2	12. 0
10	145. 8	11. 2	150. 5	11. 6
11	130. 6	10. 0	143. 7	11. 1
12	145. 1	11. 2		
MEAN	147. 6	11. 4	146. 3	11. 3

*Arranged in the same order as in tables 2 and 5, respectively.
†Estimated as 1/13 of body weight.

Discussion

In Rubin's interpretations of the results of the work with the Rous sarcoma virus, several major principles may be discerned. The findings

corroborated those of earlier investigators in demonstrating the neutralizing effects of heterologous immune serums on the Rous sarcoma virus when complement was present in the mixture of virus and antibody. An equal apparent suppression of virus activity could be accomplished, however, when serum and complement were introduced after the virus. This was a phenomenon not observed when the antibody was anti-Rous sarcoma virus serum from the chicken, a reactant requiring no complement for neutralization of the agent. Finally, the influence of the heterologous immune serum was largely reversible on dilution of the virus-serum mixtures, another behavior not seen with the homologous immune serum.

The results of previously reported experiments with the viruses of erythroblastosis and myeloblastosis can hardly be regarded as paralleling those of Rubin and the earlier investigations with the Rous sarcoma virus. Indeed, the phenomena observed with the leukemia viruses are unique in the category of filterable agents thus far investigated. Neutralization of the viruses by serums homologous with respect to both the agents and the natural host proceeds in patterns in accordance with the experience with other viruses. Highly effective neutralization of both agents is produced by antinormal chicken protein immune bodies from the rabbit, but, in contrast with the findings with the sarcoma virus, complement is not needed for the reaction.

Several major differences can be seen between the respective actions of specific immune serums from the natural host and those of the heterologous immune serums. Immune serum from the chicken has been active in neutralization of the leukemia viruses and in precipitating the respective agents, but not in binding complement with them. Anti-host protein immune serum from the rabbit has fixed complement with the myeloblastosis virus and neutralized the agent without causing the precipitation reaction. In these respects the influences of the homologous serums differed from those of the heterologous antibodies, but any evidence of a difference in the principle or mechanism by means of which neutralization was accomplished has remained obscure. Nevertheless, despite the uncertainties of the mechanisms involved, it can be concluded that the leukemia viruses differ radically from the Rous sarcoma virus in this response to antinormal chicken protein immune serum from heterologous hosts.

Still more evidence of difference between the erythroblastosis virus and the Rous sarcoma agent has been added by the present studies. Serum, in amounts equal to or greater than those producing neutralization under the usual conditions of mixture with virus and incubation, had no demonstrable influence when given separately, whether it was introduced into the host before or after virus inoculation. It has been noted (*6*) that possible intervention of complement cannot be evaded when virus and serums are injected into the host by the intravenous route. This is of no consequence for the present discussion, because no neutralization was obtained whether complement acted or not. The lack of action of the immune serum injected separately from the virus is readily understood in view of the great dilution by the relatively large volume of the circu-

lating plasma of the host. The present experiments provide unequivocal evidence of a union of virus and immune serum *in vitro* sufficiently durable to withstand dissociation by dilution either with the circulating plasma or with saline solution added after incubation outside the body. The conclusion seems unavoidable that the heterologous serum like the homologous antibody unites firmly with the virus; the serum-virus complex, whatever its nature, persists after inoculation into the host; and, consequently, the effect of the serum must be on the virus *per se* and not on the host cell.

The presentation of these results is not intended to provide a basis for criticism of the interpretations of the findings of Rubin with the Rous sarcoma virus. It is clear that the respective conditions of the experiments with that agent and with the virus of erythroblastosis were wholly different with respect to host, the tissues involved, and the mechanics of presentation of the viruses and immune serums to the host cells. The basic phenomena of neutralization were different from the beginning and, what is most important, the agents themselves were not the same. Although the viruses of myeloblastosis and erythroblastosis are antigenically related (*13*) to the agents of the avian sarcomas, they are certainly not identical with the latter in immunologic behavior, and the findings encountered with one cannot be employed as the basis for predicting the behavior and properties of another. This is particularly well exemplified by the very considerable difference between the agents of myeloblastosis and erythroblastosis. Although these viruses are obviously closely related (*2*), they are, nevertheless, dissimilar in the diseases they cause (*7*, *14–16*), the various aspects of host response (*8*, *12*, *17–19*), enzymatic activity to dephosphorylate adenosinetriphosphate (*3*, *8*, *20*), immunologic behavior in the precipitation reaction with immune serum from the chicken (*2*), and the failure of Forssman antibody to neutralize the erythroblastosis virus (*2*), in contrast to its action on the agent of myeloblastosis (*1*). As noted before, insofar as the present-day interpretations of virus neutralization are acceptable, it must be judged that a substance antigenically like host material is a constituent of that part of the erythroblastosis and myeloblastosis virus particles responsible for infectious capacity. There is no reason at the present time for construing these findings with the leukemia viruses as having any immediate bearing on knowledge of the properties of the Rous sarcoma virus.

Summary

The virus of avian erythroblastosis was neutralized by the serum from chickens immunized with formalin-inactivated virus and by antibodies produced in the rabbit by injection with normal chicken serum or saline suspensions of ground normal chicken liver and spleen. This effect was produced when serum and virus were mixed in proper proportions and incubated *in vitro* and was not dependent on the presence of complement in the mixtures. Intravenous injection of serum *after* or immediately *before* (essentially simultaneously) introduction of virus produced no neu-

tralizing effect. There was no evidence that the action of the immune serum could be reversed by dilution of incubated mixtures shortly before inoculation. These findings, together with those of earlier work, have been interpreted to indicate that the effect of the serum is on the virus particle. The results obtained with the virus of erythroblastosis were discussed in relation to recent experiments made elsewhere, which were judged to suggest that the apparent neutralizing effect of rabbit anti-normal chicken protein immune bodies on the Rous sarcoma virus was exerted directly on the host cell rather than on the virus *per se*. The sharp difference between the behavior of the respective agents, those of erythroblastosis and the Rous sarcoma, were cited.

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Biological Studies on the Rous Sarcoma Virus. VI. Variability of Recoverable Virus from Tumors Propagated by Cell Transplantation[1]

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Previous studies (*1*) have shown that, at the lower effective dose levels of Rous sarcoma virus (*2*), a high correlation exists between the dose of virus inducing a tumor and the amount of virus that can be extracted from the tumor tissue. Similar correlations have been observed also for the recovery of virus from tumors of turkeys inoculated subcutaneously (*3*) and from the brains of moribund chicks inoculated intracerebrally (*4*) with graded doses of the Rous sarcoma virus.

The present investigation was originally undertaken for the purpose of determining whether a transplantation line of Rous sarcoma could be established that would be free of extractable virus. A tumor induced in a young chicken by a minimal effective dose of Rous sarcoma virus, and confirmed by biological assay to contain an extremely small amount of extractable virus, was used to initiate a tumor transplantation line. Two sublines of the initial transplant series were also investigated, one deriving from an aberrant, rapidly growing tumor and the other from an aberrant, slowly growing tumor.

Each tumor for continuing a passage line was extracted and assayed for virus activity. Although tumors in which virus could not be demonstrated occurred sporadically, a persistent absence of virus was not observed under conditions that were compatible with maintenance of the tumor lines by cell transplantation. The bioassay results of these investigations are described herein.

Materials and Methods

Original tumor.—The Rous sarcoma for initiating the original transplantation line was taken from a titration series involving the inoculation of a frozen standard preparation of Rous sarcoma virus (CT 559) on December 1, 1954 (*5*). The donor chicken had been inoculated at 3 weeks of age with the 10^{-7} dilution of this standard virus. A small tumor was first observed on the 14th day following virus inoculation. When it was removed under aseptic conditions 6 days later, it measured $4.5 \times 3.4 \times 1.4$

[1] Received for publication June 11, 1957.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

cm. The tumor was firm and friable and in some areas was covered with greenish, discolored, hemorrhagic material. Representative fragments of the firm tumor tissue were inoculated, by a trocar, into 6 recipient chickens 6 weeks of age, and other aliquots of the fragmented tissue were taken for tests of bacteriological sterility and of extractable virus content. Bacterial culture media were inoculated immediately for the sterility tests, but the aliquot for virus assay was placed in a rubber-stoppered vial and stored in a CO_2 ice chest for 23 days prior to its use (*see 1*).

Transplantation.—All transplants were made by means of a 12-gauge trocar and involved the transfer of small fragments of solid tumor tissue measuring approximately $2 \times 2 \times 3$ mm. The transfers were made in a sterile room under aseptic conditions. Representative fragments of each tumor for passage were taken at random for the inoculation of bacteriological culture media (horse-meat infusion broth and thioglycollate). In addition, a smear taken from a random fragment was stained and examined immediately for the presence of bacteria before inoculation of the tumor into recipient chickens. Only tumor tissue shown by these tests to be free of bacteria was used for continuing the transplantation lines.

Virus assays.—The methods of storage, extraction, and bioassay of the tumor tissue were the same as those described in detail in a previous report (*1*). All tissue samples were stored in rubber-stoppered vials in the CO_2 ice chest, as previously (*1*), with the exception of the last 5 passage samples of tumors of subline C, which were in glass-sealed ampoules.

Chickens.—All chickens were of the New Hampshire Red breed and were obtained, at 1 day of age, from a local commercial source that maintains its own breeding flock. Commercial chicken feed from a constant source was used throughout the investigation. Both food and water were available at all times.

Experimental Procedures and Results

Stemline A

The "low-dose" tumor for initiating the original transplantation line is described in a preceding section (*see* Materials and Methods). In accordance with the original intent of establishing, if possible, a line that would be free of extractable virus, the procedure employed from the start was to select for continuation of the series the tumor that grew most slowly but yet did not deviate aberrantly from the remainder of the tumors of the same passage group. This selective procedure was continued throughout the investigation on the initial transplantation series or "stem line." Two subline transplant series were later established from selected aberrant tumors. At that time the initial stem line was designated as line A and the sublines as B and C.

The tumor tissues for continuing each line were stored in a CO_2 ice chest and later extracted and tested for tumor-producing activity in chicks. The extracts were prepared as previously described (*1*), each being carried through 3 successive centrifugations for the removal of intact

tumor cells. The bioassays were carried out in comparison with a standard preparation of virus and involved the use of both latent-period and tumor-incidence responses (*1*, *6*).

Detailed information on each tumor passage of line A, including the result of bioassay, is given in table 1. The first passage of the original tumor was made on December 20, 1954. Most of the subsequent passages were made at intervals of approximately 3 weeks. This represented the maximal time judged safe for a transplant of this line to remain in its host without danger of breaking through the skin and becoming contaminated. It will be noted in table 1 that the tumors of passages 16 and 17 were taken after 30 and 33 days, respectively, of growth in their hosts, instead of at the approximate 3-week interval. This did not represent a slower rate of tumor growth but was associated with retrieving tumors of a much larger size at the times indicated, for the purpose of continuing the line, since the 16th-passage tumor taken at the usual 3-week period had been found to be contaminated with bacteria. In the case of the 17th passage, transplantation for continuing the line was deferred until repeated tests on other tumors of the same group had shown that the tumor tissue transferred from the retrieved 16th passage was free of bacteria. A peculiar phenomenon was noted in the bioassay of the older tumor tissue retrieved at the 16th passage, which was not repeated on a second aliquot of the same tissue nor observed with any other passage material tested during the course of this investigation. As indicated in table 1, the response to the lowest dilution, 10^{-2}, of the first tissue sample (*a*) was entirely negative in the 10 chickens tested in the bioassay series, whereas a 100-fold weaker material (dilution 10^{-4}) produced tumors after relatively short latent periods in all 10 of the inoculated animals [the potency value for this dilution is a "single dose estimate" (*1*) based upon the average latent period]; the response to the 10^{-6} dilution was again entirely negative. The repeated test on a second sample (*b*) of the same tissue gave negative results at all 3 of these same dilutions.

At the 28th passage of stemline A, the tumor tissue was again found to be contaminated with bacteria. The investigation on this line was therefore terminated with the last passage material under biological test at the time, *i.e.*, the 25th passage.

The gross appearance of the tumors throughout the passage of line A was essentially the same as that described for the original tumor (*see* Materials and Methods). In many instances there was complete absence of hemorrhagic material; none of the tumors for continuing the line showed any gross evidence of myxomatous material.

Analyses and interpretations of the bioassay results are given on page 947.

Subline B Derived From an Aberrant, Rapid-Growing Tumor

One of the tumors resulting from the 18th passage of the stemline on November 29, 1955, grew more rapidly than the other tumors of the same group. Grossly, it was very hemorrhagic and myxomatous in

TABLE 1.—*Tumor-passage data of line A*

Passage		Age of donor chicken (*days*)†	Recipient chickens			Tumor used for passage			
								Potency of extract	
No.	Date*		Age (*days*)	No. inoculated	No. developing tumor	Time of growth in host (*days*)	Time of storage in CO_2 ice chest (*days*)	Log percent‡	Percent
(0)§	(12/1/54)	35	—	—	—	(19)‖	23	(−4. 0)	(0. 00010)
1	12/20/54	42	42	6	6	8¶	127	Neg.	(0)
2	12/28/54	50	22	3	3	21	107	−3. 0	0. 0010
3	1/17/55	42	43	5	5	—	—	—	—
			56	4	4	20	89	−3. 0	0. 0010
4	2/4/55	74	46	4	4	18	69	−1. 6	0. 025
			32	4	4	—	—	—	—
5	2/24/55	66	52	3	3	20	48	−1. 6	0. 025
			32	3	3	—	—	—	—
6	3/17/55	73	31	4	4	21	29	−0. 4	0. 40
7	4/5/55	50	38	4	4	19	8	+0. 3	2. 0
8	4/26/55	59	43	7	7	21	77	−1. 4	0. 040
9	5/11/55	58	30	3	3	—	—	—	—
			44	3	3	15	56	−0. 6	0. 25
			58	3	3	—	—	—	—
10	6/1/55	64	51	3	3	—	—	—	—
			79	3	3	20	36	−2. 8	0. 0016
11	6/21/55	99	43	3	3	—	—	—	—
			57	3	3	20	16	−1. 4	0. 040
12	7/11/55	77	35	3	3	20	38	−2. 8	0. 0016
			49	2	2	—	—	—	—
			63	2	2	—	—	—	—
13	8/1/55	56	42	6	6	21	17	+0. 3	2. 0
14	8/22/55	63	49	6	6	21	65	Neg.	(0)
15	9/12/55	70	56	8	8	21	12	Neg.	(0)
16	10/6/55	86	51	10	7	30**	8	††*a* 10^{-3}, neg.; 10^{-4}, +0.2; 10^{-6}, neg.	(0); 1. 6; (0)
							22	††*b* Neg.	(0)
17	11/8/55	84	29	8	7	33**	42	−1. 9	0. 013
18	11/29/55	50	50	6	6	21	20	Neg.	(0)
19	12/21/55	72	30	10	10	22	124	+1. 4	25. 0

20	1/13/56	53	53	10	10	23	98	Neg.	(0)
21	2/7/56	78	36	7	6	25	78	−2. 1	0. 0080
22	2/28/56	51	43	8	8	21	68	+0. 7	5. 0
23	3/20/56	64	36	8	8	21	53	−0. 6	0. 25
24	4/9/56	56	56	8	8	20	35	+2. 2	158. 5
25	4/27/56	74	60	7	7	18	14	+0. 9	8. 0

*Month/day/year.

†Age at time tumor was taken.

‡Relative to standard virus (CT 629) used for the assay. Log percent potency=log-potency ratio (*M*) plus 2.0(*I*). Potency of standard=100 percent or 2.0 log percent. All values have been rounded to the first decimal.

§Original tumor (*see* Materials and Methods).

‖Time from inoculation of virus.

¶Tumor taken earlier at a smaller size than in subsequent passages. Not a faster growing tumor.

**Tumor taken later at a larger size than for other passages. Not a more slowly growing tumor—*see* text, p. 943.

††*See* text, p. 943.

appearance. This tumor was used to start a parallel subline, designated subline B, which began with the 19th passage on December 13, 1955. Subsequent passages were made at 1-week intervals, the largest and most rapidly growing tumor of each transfer group being selected for continuing the subline. The selective procedure was therefore the extreme opposite of that described above. All tumors of subline B were grossly hemorrhagic and myxomatous. Because of the short interval between transfers and the resultant rapid accumulation of tumors to be assayed, the subline was discontinued after the 31st passage (the 13th serial transfer of the subline), at which time the extract was equal in virus potency to that of the reference standard. The experimental details and results are recorded in table 2.

TABLE 2.—*Tumor passage data of subline B**

Passage		Age of donor chicken (*days*)	Recipient chickens			Tumor used for passage			
								Potency of extract	
No.	Date		Age (*days*)	No. inoculated	No. developing tumor	Time of growth in host (*days*)	Time of storage in CO_2 ice chest (*days*)	Log percent	Percent
(18)†	(11/29/55)	—	—	—	—	—	—	—	—
19	12/13/55	64	36	8	8	14	21	+0.4	2.5
20	12/20/55	43	27	10	10	7	14	+0.7	5.0
21	12/27/55	34	36	8	8	7	7	+1.6	40.0
22	1/3/56	43	29	6	6	7	56	+1.4	25.0
23	1/10/56	36	36	6	6	7	49	+0.4	2.5
24	1/17/56	43	29	6	6	7	42	+0.5	3.2
25	1/24/56	36	36	6	6	7	35	+1.3	20.0
26	1/31/56	43	29	7	7	7	28	+1.6	40.0
27	2/7/56	36	36	8	8	7	21	+0.5	3.2
28	2/14/56	43	31	6	6	7	14	+1.9	79.5
29	2/21/56	38	36	8	8	7	7	+1.6	40.0
30	2/28/56	43	29	8	8	7	87	+1.4	25.0
31	3/6/56	36	36	8	8	7	80	+2.2	158.5

*See footnotes table 1.
†Eighteenth passage of original line A which gave rise to subline B.

Subline C Derived From an Aberrant, Slow-Growing Tumor

Of 8 chickens inoculated with stemline A tumor tissue at the 17th passage, one failed to develop a tumor and another showed only a small hard tumor on the 21st day, whereas the tumors of the remaining chickens were quite large and of the usual size at time of retransfer. The small tumor was observed to be relatively slow-growing, and on the 33rd day it was taken to establish a second subline. This began with the 18th passage on December 13, 1955, and was designated subline C. In order to favor the chances of establishing a "virus-free" tumor line, slightly older recipient chickens were used, when available, than for passages of the 2 other tumor lines. Also, the most slowly growing tumor of the group was always used for the transfer at each passage. All tumors for con-

tinuing subline C grew relatively slowly and showed no gross evidence of either hemorrhagic or mucoid material. In many instances they appeared slightly yellowish in color. This subline was automatically terminated after the 30th passage (the 13th serial transfer of the subline) since none of 9 chickens inoculated with the most slowly growing tumor of this passage group (*i.e.*, at the 31st passage) developed a tumor. The experimental conditions and results are summarized in table 3.

Analysis and Interpretation of Results

The data reported here were obtained under 3 different conditions of selection of tumors for the continuation of transplantation lines. The yield of virus from Rous sarcoma tissue has been repeatedly associated with the rate of growth of tumors in their hosts (*1*, *7*, *8*), and it was for this reason that selections were made on a basis of relative growth rates of tumors within the respective lines.

Transplantation line A and subline C were directed toward the establishment of "virus-free" tumor lines and involved the selection of the more slowly growing tumors. In all instances the selection was relative to the other tumors within the same passage group. In line A the slowest growing tumor, except for aberrant variants, was used at each passage. In subline C the slowest growing tumor was always used regardless of the fact that it may have differed significantly from all other tumors within the same passage group.

The purpose of the investigation on subline B was the extreme opposite, namely, demonstrating in a systematic study the fact already well known from previous experience in this as well as in other laboratories (*1*, *7*, *8*) that the highest yields of virus are obtained from the most rapidly growing tumors. Advantage was taken of a rapidly growing variant, which occurred spontaneously in the stemline series A, for retesting this correlation.

The bioassay results detailed in tables 1 to 3 are shown graphically in text-figure 1. The potency values are relative to a frozen standard preparation of virus (CT 629) and are plotted as percentages on a log scale. It is seen in charts A and C of this figure that selection of the more slowly growing tumors did not result in consistently low yields of virus in the tumor-tissue extracts nor in the establishment of lines that were free of extractable virus. On the contrary, the virus yields showed extreme fluctuations between successive passages as well as broad undulatory variations from time to time throughout the course of the respective investigations. Any over-all regression of the observed potency values with progressive passages would be entirely without significance in the case of tumor line A and subline C, because of the wide fluctuations in results and the consequent extreme experimental errors of the regression coefficients.

Twenty-four percent of the tumors of line A and 46 percent of those of subline C failed to yield demonstrable virus on extraction and are therefore listed as "negative." Since the virus "reappeared" in later passages after 1 to 3 transfers, during which it was not detectable, it is evident that the

TABLE 3.—*Tumor passage data of subline C**

Passage		Age of donor chicken (*days*)	Recipient chickens				Tumor used for passage			
					No. developing tumor		Time of growth in host (*days*)	Time of storage in CO_2 ice chest (*days*)	Potency of extract	
No.	Date		Age (*days*)	Corrected no. inoculated†	Growing tumor	Small stationary nodule‡			Log percent	Percent
(17)§	(11/8/55)									
18	12/13/55	84	50	6	5	0	35	120	−2. 1	0. 0080
19	1/17/56	85	47	8	6	2	35	85	Neg.	(0)
20	2/21/56	82	50	8	3	3	35	67	Neg.	(0)
21	3/26/56	83	56	8	5	0	33	66	−1. 6	0. 025
22	4/27/56	88	60	4	2	0	32	47	−0. 08	0. 83
23	5/28/56	92	49	8	2	6	32	70	Neg.	(0)
24	6/28/56	70	66	8	6	0	31	38	−0. 5	0. 32
25	7/30/56	98	56	8	6	2	32	7	Neg.	(0)
26	8/30/56	87	59	8	5	0	31	36	Neg.	(0)
27	10/1/56	91	50	8	3	0	32	30	−3. 5	0. 00032
28	10/30/56	79	50	10	4	2	29	44	−0. 2	0. 63
29	11/29/56	80	51	10	1	0	30	14	+1. 9	79. 5
30	12/28/56	80	39	10	10	0	—	—	—	—
			53	6	6	0	29	31	Neg.	(0)
31	1/28/57	84	56	9	0	0	‖	‖	‖	‖

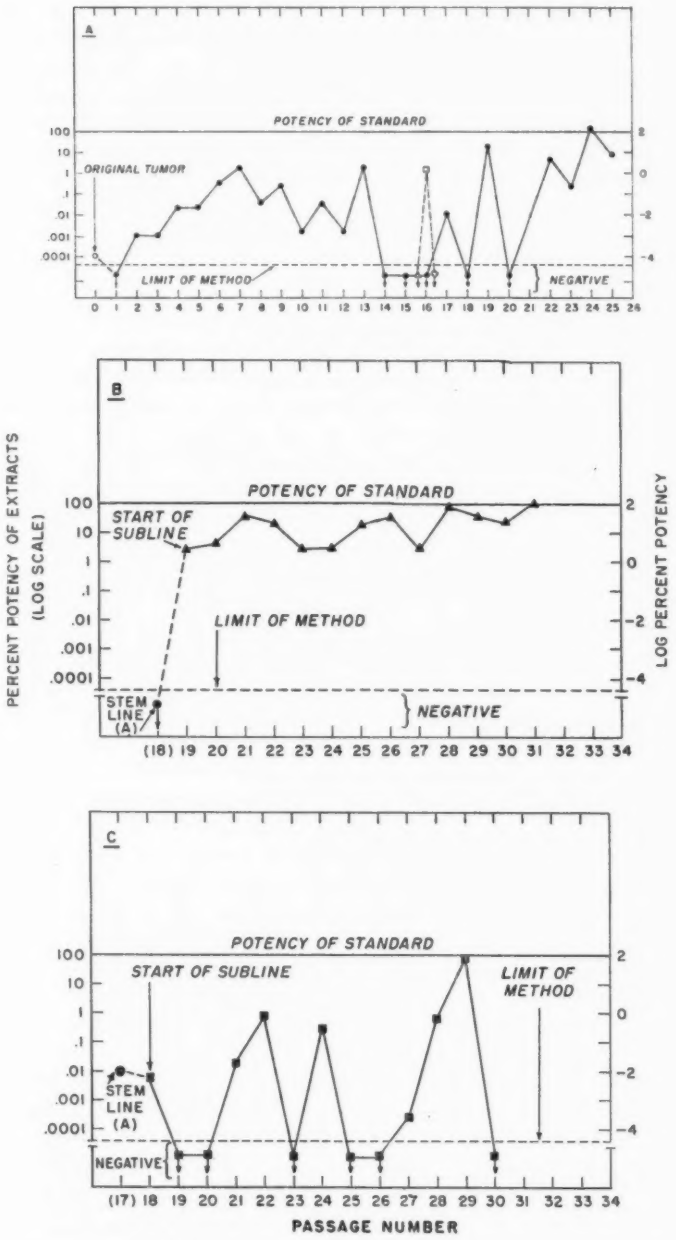
**See* footnotes table 1.

†Two chickens died during the experimental period in each of passage groups 18 and 22. The number inoculated was 2 more than the "corrected" total, in these instances.

‡Probable small tumor, but diagnosis uncertain.

§Seventeenth passage of original line A which gave rise to subline C.

‖ No tumors developed from transplants of the 30th passage tumor tissue.



TEXT-FIGURE 1.—Relative potencies of extracts of tumors used for successive passages. Charts A, B, and C represent tumor line A and sublines B and C, respectively (*see* text).

very small quantities of virus carried by such "negative" tumors were below the limits of sensitivity of the biological tests employed. It should be re-emphasized in this connection (*see also 1*) that the "limit" of the assay method is a statistical variable and that it is a function of the most susceptible chicken that happens to be included, by chance, within a particular test group. When the number per dilution group is small (*e.g.*, 10), therefore, fluctuations of the order of a $\log_{10}$ interval or more in the "limit" of the method are to be expected. This is not of critical importance in investigations of the present type in which decreases in potency of as much as 5 to 7 $\log_{10}$ intervals could be registered before the "limit" was reached.

The rapidly growing variant that gave rise to subline B is seen from text-figure 1 (chart B) to have contained a relatively high concentration of extractable virus (only 1.6 $\log_{10}$ intervals below that of the strong reference standard). The further passage of this tumor through selection of the most rapidly growing tumor at each transfer not only maintained the virus concentration of the passage line at a high level but also resulted in a progressive upward trend of the virus concentration with successive passages. The trend is statistically significant in comparison with the variation about a linear regression line ($0.05 > P > 0.01$). What is even more striking is the relatively narrow range of variation, including the regression, of the potency yields of tumors of subline B in comparison with those of the other 2 lines.

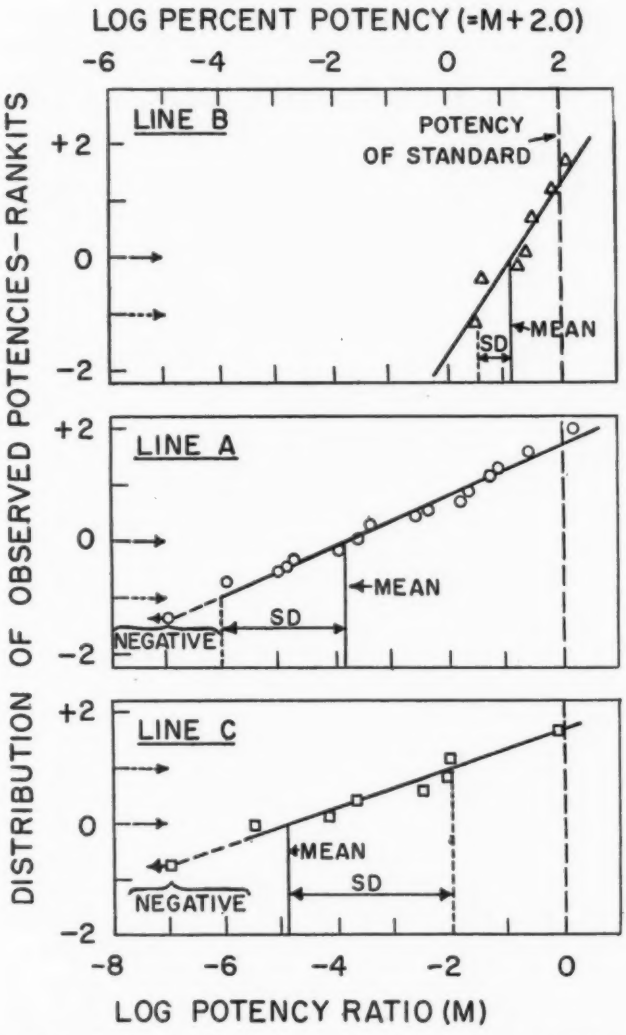
Since the "negative" tumors of line A and subline C obviously contained very small quantities of virus below the limits of sensitivity of the biological test method, they cannot be considered as representing true zero estimates of extractable virus, although they did approach zero (*i.e.*, $-\infty$ on a logarithmic scale). For this reason the arithmetic mean and the standard deviation of the individual log-potency estimates could not be derived directly from the data of line A and subline C. These parameters were estimated, however, by the method of rankits involving procedures appropriate for truncated distributions (*9–11*). The graphic rankit analyses[3] for all 3 tumor lines[4] are illustrated in text-figure 2, in the order of the mean quantity of extractable virus, *i.e.*, B, A, and C.

The results of the graphic analyses are summarized in table 4, which also includes for each line the average time interval between successive passages—exclusive of the instances in which there was deviation from the usual procedures owing to bacterial contamination.

Although the latter averages are accurate representations of the actual mean time intervals between passages, they do not represent precise measures of tumor growth rates since the tumors were not all taken at the same standard size. The criterion established for harvesting tumors of stemline A was that they be allowed to remain in their hosts as long as

[3] Average rankit values were plotted against the averages of the corresponding potency estimates for groups of 2 or more observed values that differed by no more than 0.2 $\log_{10}$ interval. (For a discussion of the method of rankits in the analysis of virus bioassay data, *see 11.*)

[4] A similar analysis of the data of line A through the 22nd passage was made previously and included in a recent report (*8*). The mean given therein differs slightly from the present value based upon all data through the 25th passage.



TEXT-FIGURE 2.—Illustrating the graphic rankit method for estimating the mean and standard deviation of observed virus-potency values. The charts represent tumor lines of the same designations (*see* text) and are arranged in order of the mean quantity of extractable virus (*i.e.*, B, A, and C).

possible without danger of rupturing through the skin. For convenience in scheduling the use of a sterile room on certain days of the week, the transplantation schedules were adjusted approximately to multiples of 1 week. An interval of 3 weeks was found to be entirely safe for tumors of line A. After the first passage in subline B, the tumors enlarged so rapidly (due, in part, to the accumulation of mucoid fluid) that it was not

judged safe to allow the tumors to remain in their hosts for a 2-week period. They were therefore taken at weekly intervals, although, on an average, they were somewhat smaller at the time of harvest than tumors of line A. The tumors of subline C were taken when they were approximately the same size, on an average, as those of line A. It is possible, however, that these more slowly growing tumors could have been allowed to grow to an even larger size without danger of rupturing. The growth time found to be appropriate for subline C was approximately 4 weeks. In spite of the differences in criteria and in final tumor size at time of harvest, the average intertransplantation intervals in table 4 reflect major differences in the rates of growth of tumors of the different lines. In fact, it was due to such differences that these technical variations entered into the experimental data. The average time intervals may therefore be used as rough indicators of the grossly apparent differences in tumor growth rates.

TABLE 4.—*Summary of means and standard deviations of log-potency-ratio estimates* (M) *derived by graphic methods* (see *text*)*

Tumor line	Mean† log-potency ratio (*M*)	Standard deviation (Log_{10} *units*)	Average interval‡ between successive transplants	
			Days	100/Days
B	−0.9	0.6	7	14.3
A	−3.8	2.2	20	5.0
C	−4.9	2.9	32	3.1

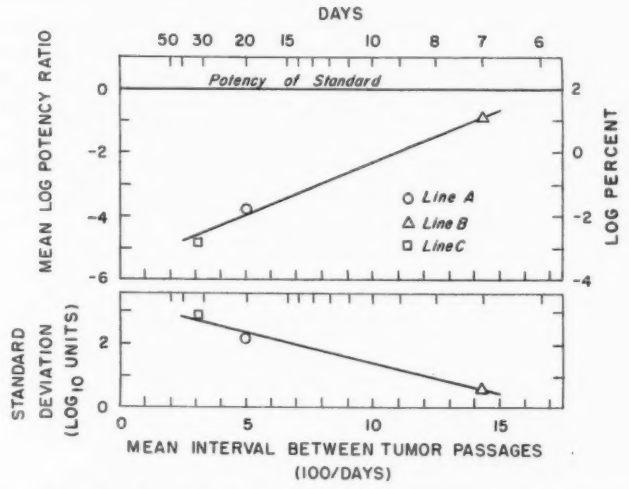
*All values have been rounded to the first decimal.

†Mean of log-potency-ratio values (*M*) where the potency of the standard is considered to be unity and its logarithm is therefore zero.

‡Exclusive of the special instances in which the interval was altered for reasons given in the text.

The relationship of the mean and the standard deviation of observed virus-potency values to the average intertransplantation interval is shown in text-figure 3. It is apparent that both the mean quantity of recoverable virus and the standard deviation of the individual potency estimates about their means were significantly correlated with the intertransplantation interval. To the extent that this interval reflects the differences in growth rate, therefore, the average amount of extractable virus and the extent of the variation among individual tumors in their yields of virus are shown to be related, on a statistical average basis, to tumor growth rate.

These findings confirm the experience of previous investigators (*see 1, 7, 8*) that the yield of extractable Rous sarcoma virus is related, on an average, to the tumor growth rate. The additional information revealed is that the variation (in terms of standard-deviation increments) of individual tumors is approximately 5 times greater on a logarithmic-potency scale among slow-growing tumors of low average virus yield than among rapid-growing tumors of relatively high average yield. This amounts to



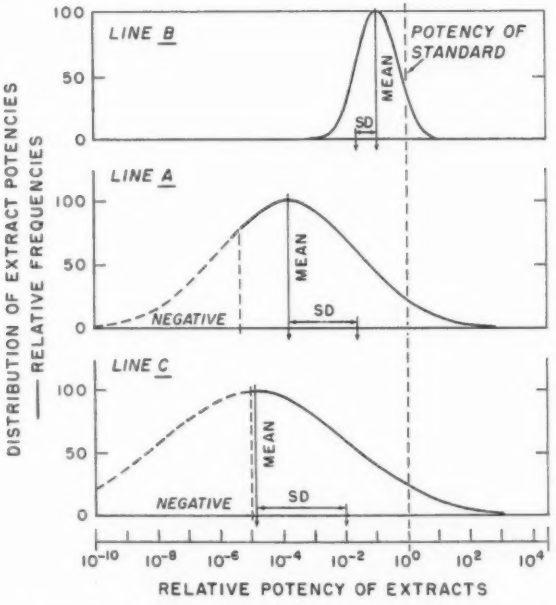
TEXT-FIGURE 3.—Showing the relationship of the mean quantity of extractable virus (*upper chart*) and of the standard deviation of individual tumors (*lower chart*) to the average intertransplantation interval (a rough indicator of tumor growth rate).

a difference in variability of a factor of 10^5 in terms of arithmetic potency values. The extreme differences in variability are illustrated diagrammatically in text-figure 4, which shows normal distribution curves having the same means and standard deviations as those estimated in text-figure 2, and summarized in table 4. The fairly good approximations of the results to straight-line relationships in text-figure 2 permit this use of normal curves as fairly representative approximations. With the relatively small amount of data available, however, and in view of the significant regression included in the variations within subline B, it is not intended to imply that exact normal distributions have been actually demonstrated for these data. Rather, the curves are employed as approximate representations of the respective distributions in order to depict more clearly the practical significance of the order of magnitude differences in variability, *i.e.*, in standard deviation values.

Discussion

Although the original purpose of establishing a "virus-free" transplantation line of the Rous sarcoma was not accomplished, the quantitative bioassay data acquired during the attempt to derive such a line are of considerable value in elucidating the relationships between tumor properties and virus yield. It has been the common experience of all who have worked with the Rous sarcoma that the best yields of virus are obtained from the most malignant and most rapidly growing tumors, whereas tumors of the more benign and slow-growing type frequently yield little or no virus on extraction. The fact that the correlation was not absolute

and that "strangely unaccountable exceptions" (*12*) to the rule sometimes occurred seemed baffling to some of the earlier investigators (*see 1, 7*). The tumor lines studied in the present investigation were maintained through selections for successive passages of either those tumors that grew most slowly (line A and subline C) or those that grew most rapidly (subline B). Within each of the 3 lines, the selective procedure resulted in the establishment of a transplant series of tumors that grew at roughly the same rate, *i.e.*, the growth rates were widely enough separated so that there was no overlap among individual *selected* tumors of the different lines.



TEXT-FIGURE 4.—Normal curves having the same means and standard deviations as those estimated in text-figure 2 and summarized in table 4. The chart designations conform to those of the respective tumor lines. They are arranged in order of magnitude of the mean—*see* legend for text-figure 3 and text.

With this control of the growth-rate factor, the extractable virus content was found to be uniformly high, and even to increase on successive passages when the tumors were those of the most rapidly growing type (subline B). Apart from the significant increase in virus content on passage, the variation among individual tumors of subline B (*i.e.*, the variation about a linear regression line) was not significantly greater than what might be expected from the bioassay error alone. These findings are of considerable practical importance since they define the biological conditions under which potent and highly reproducible virus source materials can be maintained in the laboratory (*i.e.*, through the selection

and use of only those tumors which grow most rapidly), and they indicate procedures by which the virus content of tumor tissue can be increased, at least to some extent. An interim report on a similar investigation involving the serial passage of virus rather than of tumor cells was included in a recent discussion (*13*).

Contrary to the findings with the rapid-growing tumors of subline B, the slow-growing tumors of line A and subline C showed tremendous variations in extractable virus content. The virus yields were not uniformly low, as might be expected if growth rate were the only factor of importance, but they varied over the entire range from potencies as high as those recorded for subline B, as well as for the strong reference standard, to no demonstrable virus at all. Within the range in which virus activity was measurable, the recorded differences in virus quantity extended over approximately 6 $\log_{10}$ intervals. When it is considered that the "negative" tumors represented amounts of virus below the limit of the method rather than actual absence of the virus, it is clear that the variation from tumor to tumor was in excess of 6 $\log_{10}$ intervals. Some other factor, or factors, besides the rate of increase in over-all tumor size must be operative therefore in determining the quantity of extractable virus. The presence or the effectiveness of such factors appears, however, to be related in some way to tumor growth rate.

Although exaggerated by the selective procedures employed, the low virus yields and the lack of reproducibility observed among the slow-growing tumors of line A and subline C are reminiscent of the similar findings reported for the Rous sarcoma during the earlier years of its propagation and study in the laboratory (*see 7* for review). The fact that such tumors could be readily duplicated through the use of a high dilution (low dose) of one of the strongest virus preparations available at the present time indicates that the phenomenon is a function of virus quantity *per se*, and that the more potent virus materials now available simply represent increases in virus quantity that have been achieved through laboratory passage, rather than an alteration of the original virus to a more "virulent" type.

Other observations that recapitulate the findings of earlier investigators who worked with relatively "low-dose" Rous sarcomas were: 1) the "temporary disappearance of filterability of tumors" (*see 1, 7*); and 2) the sudden change from a nonhemorrhagic to a very hemorrhagic type of tumor in a transplantation line. Although the present extracts were not filtered, filtrates of them would be expected to have been weaker in virus activity than the potencies observed, since some loss in virus usually occurs during filtration (*see 7*). The run of "negative" tumors in 3 successive passages (14th through 16th) of line A is therefore comparable to the "nonfilterable phase" frequently recorded in earlier literature. The rapidly growing variant tumor of stemline A, which gave rise to subline B, was very hemorrhagic throughout and was comparable to the variation observed by Rous and Murphy (*14*) "in the eighth tumor generation, some fifteen months from the time of the original transplantation."

Summary

A tumor induced with a low dose of Rous sarcoma virus was used to initiate a cell transplantation line that was further maintained through selection of the most slowly growing tumor (except for aberrant variants) for each successive cell passage. Subline transplant series were also established from an aberrant, rapid-growing tumor and from an aberrant, slow-growing tumor. Attempts to produce a "virus-free" line of the tumor through selection with respect to tumor growth rate were unsuccessful.

The quantitative bioassay data associated with these investigations are presented and an analysis is made of the relationship between tumor growth properties and the yield of extractable virus.

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Walker Carcinosarcoma 256 Tissue as a Dietary Constituent. I. Stimulation of Appetite and Growth in the Tumor-bearing Rat [1]

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Weight loss in rats bearing Walker carcinosarcoma 256 may follow a decrease in food consumption. Anorexia, a common phenomenon among hosts bearing either clinical or experimental cancers, appears significant in the deterioration of the host. Attempts to circumvent reduced food intake through gavage have not been completely successful in eliminating weight loss from the relatively normal tissues of the cancerous rat (*1*).

Little is known about factors concerned with the maintenance of food consumption, but two observations seem especially pertinent:

1) Deletion of an "essential" nutrient from the diet of an experimental animal causes the animal to decrease its food intake.

2) Richter's work (*2*) on self-selection of diet among experimental subjects with induced biochemical defects indicates the ability of such subjects to choose those dietary ingredients that will heal or compensate for the specific deficiency when they are presented with a variety of alternate diets. In fact, Richter observed that tumor-bearing rats often ate the cancers from their pen mates, or even their own tumors. We have observed this curious behavior among cancerous experimental animals and, also, that the normal tissues are not eaten under these circumstances.

Moreschi (*3*) developed a thesis that death of a tumor-bearing animal was due to the withdrawal by the neoplasm of a specific autogenous nutrient essential to life. Experiments are reported here in which this thesis was tested by studying the influence of Walker carcinosarcoma 256 as a dietary ingredient for rats with this same tumor growing progressively in their subcutaneous tissues. Similar studies have been reported previously (*4–6*).

Materials and Methods

The Walker 256 tumor tissue for feeding was obtained in the following manner: Small pieces of tumor were implanted subcutaneously in Sprague-Dawley rats each weighing 150 to 200 gm. At the end of 2 or 3

[1] Received for publication July 9, 1957.

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[3] The authors are indebted to Mr. Nathan Mantel of the Biometry Branch, National Cancer Institute, for his advice and assistance.

[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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weeks, the animals were sacrificed and the tumor was dissected out. The individual tumors varied in wet weight from 50 to 125 gm. When grown to this size, the Walker 256 tumor consists of a shell of firm, viable tissue surrounding a necrotic core. No attempt was made to separate the viable from the necrotic portions.

The tumors were ground at once in a meat grinder and placed in round-bottom flasks that were spun rapidly and frozen at −72° C. The tissue was dehydrated under reduced pressure. The dried tumor was removed and ground in a corn mill. The final product was a fine nonhygroscopic powder. This material was incorporated into the experimental diet designated T-24 (table 1). T-24 was compared in feeding experiments with 2 casein control diets, C-25 and C-20. The composition of these 2 diets is also shown in table 1.

Male Sprague-Dawley rats, each approximately 4 weeks old and weighing 55 to 70 gm., were maintained on a Purina chow diet and given aureo-

TABLE 1.—*Composition of diets**

Components	C-25 (*percent*)	C-20 (*percent*)	T-24 (*percent*)
Casein (vitamin-free)	25. 0	20. 0	—
Walker 256 tumor (lyophilized)	—	—	24. 0
Cornstarch	31. 9	36. 4	32. 4
Sucrose (vitamin-fortified) †	15. 0	15. 0	15. 0
Crisco	24. 0	24. 0	24. 0
Salt mixture (7)	4. 0	4. 0	4. 0
DL-methionine	—	0. 5	0. 5
Choline	0. 1	0. 1	0. 1
	100. 0	100. 0	100. 0

*Each animal received weekly 0.5 ml. of cod-liver oil.

†Vitamin supplement in fortified sucrose (per 1,000 gm. diet): thiamin HCl, 5 mg.; riboflavine, 10 mg.; pyridoxine HCl, 5 mg.; calcium pantothenate, 5 mg.; niacin, 10 mg.; *p*-aminobenzoic acid, 300 mg.; inositol, 1,000 mg.; folic acid, 5 mg.; biotin, 1 mg.; vitamin K, 2 mg.; α-tocopherol acetate, 500 mg.; vitamin B_{12}, 0.8 mg.

mycin in their drinking water at a concentration of 8 gm. per gallon. This precautionary measure, previously observed to be effective in preventing respiratory infections, was continued for 2 weeks only. At a suitable time following the antibiotic treatment, depending on the nature of the experiment, the animals were inoculated subcutaneously in the right dorsal quadrant with the Walker 256 tumor.

After transfer to an experimental diet, each animal was housed individually in a wire mesh-bottomed cage through which the excerta were allowed to drop. Water and food were administered *ad libitum*. Daily food intakes and weights were recorded.

Two different experiments were performed. In experiment A, a study was made of the changes in cachectic tumor-bearing rats when they were transferred from a casein diet to the tumor diet. Since previous experience with C-25 had shown that a high proportion of tumor-bearing rats showed a decrease in both food intake and weight on this diet, C-25 was the casein control in this experiment.

In experiment B, the tumor diet was consumed throughout the period of growth of the tumor and the effects of this regimen were compared with the effects of C-20. In this case, C-20 was the basal diet since it provided better maintenance of food consumption and weight in tumor-bearing rats than C-25. T-24 and C-20 were approximately isocaloric and contained equal amounts of nitrogen, as determined by bomb calorimetry and micro-Kjeldahl procedure.

Experiment A

Forty-five rats each weighing 120 to 140 gm. were allowed to eat the C-25 diet for 4 days prior to tumor transplantation. The animals, bearing progressively growing tumors, were continued on this regimen until they showed a pronounced reduction in food consumption and a corrresponding loss in weight for 3 to 6 consecutive days. Then they were transferred to the T-24 diet.

Of the 45 animals originally started on this experiment, 11 were available for these growth studies. Among the remainder, a few had to be discarded early because of respiratory infections or because the tumors became ulcerated. A few rats continued to eat the basal diet normally and without weight loss until death. The food consumption of a few animals ingesting the basal diet dropped for 2 or 3 days but returned to almost normal without supplementation. Finally, a few rats that showed a decrease in food intake and weight failed to eat the basal diet, continued to lose weight, and died in a few days.

Experiment B

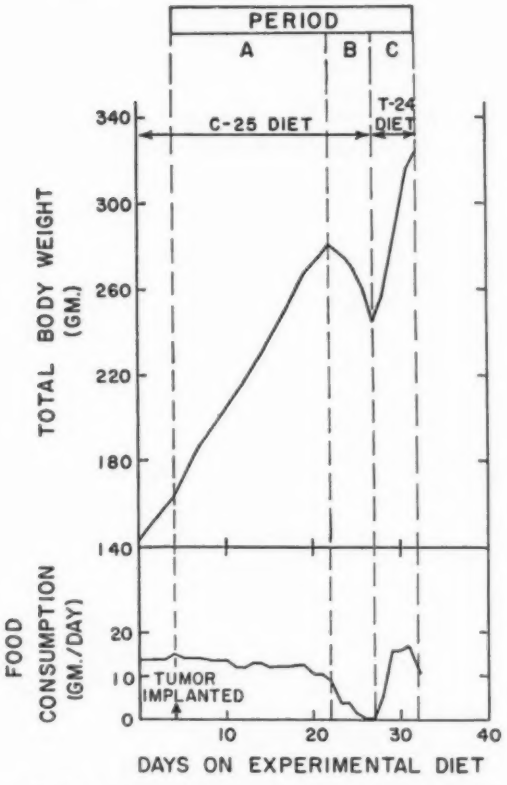
Seven days following tumor transplantation, 26 rats each weighing 173 to 218 gm. were selected and divided into 2 groups to be placed on either the C-20 or T-24 diet. The 2 groups contained rats of similar weights. Throughout the period following transfer to the respective diets, the tumors were measured with calipers along their 3 principal axes.

On the 27th day, 7 rats on the T-24 diet and 6 on C-20 still survived, and they were sacrificed at this time by cervical dislocation. The other rats had been discarded because their tumors had ulcerated.

Results

Experiment A

The growth and food-intake curves of a typical rat from this experiment are shown in text-figure 1. During period A, when the tumor-bearing animal was on C-25, it gained weight at a constant rate and maintained a fairly constant level of food intake. During period B, the rat was still on the C-25 diet but showed a marked drop in food consumption and a parallel drop in body weight. At the end of period B, the rat was transferred to the T-24 diet. A prompt increase in food intake and a concomitant increase in weight was observed in period C.



TEXT-FIGURE 1.—Body weight and food intake of a typical tumor-bearing rat (#2460) ingesting the C-25 diet followed by the T-24 diet.

The data for each of the 11 rats during periods A, B, and C are shown in table 2. In period A, the number of days prior to decrease in food intake and body weight covered a wide range (18–32 days), indicating the variability among individual rats in their response to the presence of the tumor.

In earlier experiments when an animal failed to eat, the original C-25 diet was continued, but 500 mg. of tumor tissue were given as a separate supplement. Each day the rat would consume the supplement but would not necessarily eat the basal diet. However, if the animal ate the supplement on 4 consecutive days, it would invariably start to consume the basal diet.

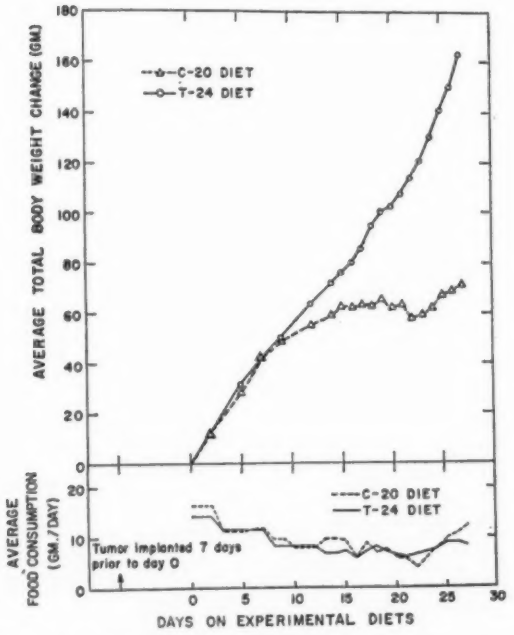
Experiment B

(*a*) *Changes in Total Body Weight and Tumor Size with Time*

Text-figure 2 shows the growth and the food consumption of rats eating either the C-20 or T-24 diet during the 27-day period. To eliminate the effect of variability in the initial weights at the time of transfer to the

TABLE 2.—*Effect of T-24 diet on weight change and food intake of anoretic rats bearing the Walker 256 transplanted tumor*

Rat no.	Period A			Period B			Period C		
	Days on C-25	Average food intake (*gm./day*)	Average weight change (*gm./day*)	Days on C-25	Average food intake (*gm./day*)	Average weight change (*gm./day*)	Days on T-24	Average food intake (*gm./day*)	Average weight change (*gm./day*)
2426	18	9. 2	4. 2	5	2. 2	−4. 8	7	10. 3	12. 9
2429	24	11. 6	6. 5	5	2. 2	−6. 6	4	10. 5	10. 8
2434	18	9. 6	4. 7	5	3. 0	−3. 8	5	8. 4	9. 4
2435	21	10. 0	5. 1	5	2. 8	−5. 0	15	10. 4	9. 3
2441	21	11. 5	5. 9	5	1. 2	−8. 2	4	15. 5	24. 8
2446	21	13. 9	7. 8	5	2. 6	−7. 0	3	10. 7	10. 7
2460	18	12. 6	6. 5	5	1. 6	−7. 2	5	13. 0	16. 0
2461	22	11. 2	5. 0	6	2. 2	−6. 5	3	11. 7	16. 3
2303	32	12. 3	6. 3	6	3. 5	−6. 0	3	15. 0	19. 7
2306	29	12. 1	4. 8	3	0. 0	−8. 7	7	10. 6	13. 4
2309	27	11. 6	4. 9	6	1. 8	−6. 5	6	12. 0	17. 2
Mean.....	.....	11. 4	5. 6		2. 1	−6. 4		11. 6	14. 6



TEXT-FIGURE 2.—Total body-weight changes and daily food consumption of tumor-bearing rats ingesting either the C-20 or the T-24 diet.

diets, the total body weight (rat plus tumor) has been calculated as the increment above the initial weight. The average of these increments has been plotted.

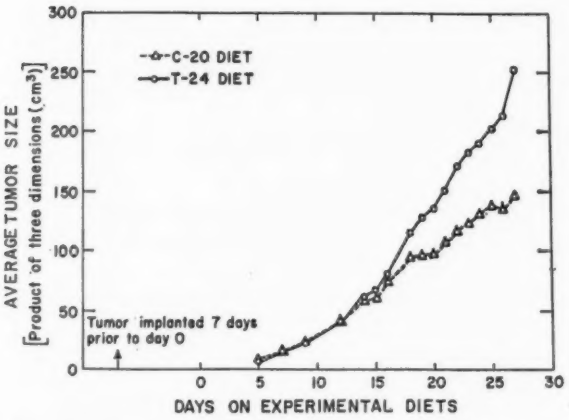
It can be seen that the T-24 diet permitted a far more rapid increase in average total body weight than did the C-20 diet. This increase in weight cannot be attributed to differences in food consumption, for the rats ate equally well on the 2 diets.[5]

Text-figure 3 presents the average growth of tumor on the 2 diets. Size of the tumor has been plotted as the product of the 3 tumor dimensions. It is apparent that increase in mass of the tumor in rats on the T-24 diet can account for at least part of the increased gain in total body weight by those rats.

(*b*) *Final Weight Changes*

The weight changes in each animal and its daily food consumption from the time it was placed on the synthetic diet until death are given in table 3. Included in this table are data from 6 rats (3 on C-20 and 3 on T-24) in an earlier experiment. These rats were sacrificed after eating the experimental diet for 28 days. Change in carcass weight (table

[5] Young rats without tumors grow equally as well on the T-24 as on the C-20 diet. These results will be reported in a subsequent paper.

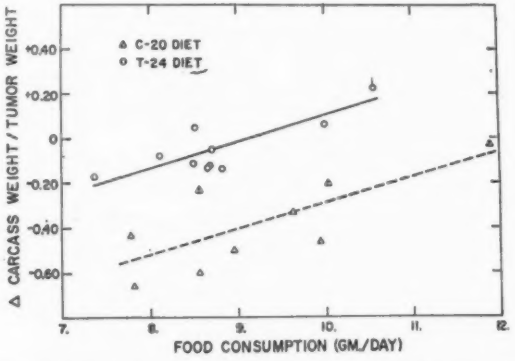

C-20 DIET
T-24 DIET
AVERAGE TUMOR SIZE
[Product of three dimensions (cm³)]
Tumor implanted 7 days prior to day 0
DAYS ON EXPERIMENTAL DIETS

TEXT-FIGURE 3.—Growth of Walker carcinosarcoma 256 in rats ingesting either the C-20 or the T-24 diet.

3) was determined by subtracting the tumor weight at death from the change in total body weight.

A comparison of the effects of the 2 diets (last line of table 3) shows that the rats on the T-24 diet gained an average of 91 gm. more in total body weight than that exhibited by the C-20 controls. Of this increase, 61 gm. was tumor weight and 30 gm. was change in carcass weight.

The relationship between Δ carcass weight/tumor weight and food consumption (gm./day) is shown in text-figure 4. Data are included from all the rats of table 3. Text-figure 4 shows that on both diets there was an increase in Δ carcass weight/tumor weight with increased food consumption, as might be expected. The rats on the T-24 diet showed a shift toward more positive values of Δ carcass weight/tumor weight for any given value of dietary intake. This result also might be expected



TEXT-FIGURE 4.—Relationship between Δ carcass weight/tumor weight and food consumption of rats ingesting either the C-20 or the T-24 diet.

TABLE 3.—*Food intake and weight changes (tumor and carcass) of rats ingesting either the C-20 or the T-24 diet*

Diet	Rat no.	Food consumption (*gm./day*)	Initial body weight (*gm.*)	Final total body weight (*gm.*)	Change in total body weight (*gm.*)	Tumor weight (*gm.*)	Change in carcass weight (*gm.*)
(I) C-20	2652	9. 0	212	273	+61	123	−62
	2654	11. 9	189	309	+120	124	−4
	2656	9. 6	218	309	+91	136	−45
	2658	7. 8	217	256	+39	114	−75
	2664	9. 9	198	249	+51	95	−44
	2666	7. 8	186	250	+64	112	−48
	2366*	8. 6	188	267	+79	103	−24
	2376*	8. 6	244	280	+36	89	−53
	2384*	10. 0	216	316	+100	126	−26
	Mean ± S.E.†	9. 2 ± 0. 5	208 ± 7	279 ± 8	+71 ± 9	113 ± 5	−42 ± 8
(II) T-24	2645	8. 8	205	404	+199	230	−31
	2649	8. 5	182	359	+177	169	+8
	2657	8. 7	214	385	+171	196	−25
	2661	8. 7	208	367	+159	181	−22
	2663	10. 6	202	327	+125	102	+23
	2665	8. 1	187	321	+134	146	−12
	2669	7. 4	174	350	+176	212	−36
	2321*	10. 0	212	367	+155	146	+9
	2373*	8. 7	222	382	+160	168	−8
	2379*	8. 5	236	405	+169	190	−21
	Mean ± S.E.†	8. 8 ± 0. 3	204 ± 6	367 ± 9	+162 ± 8	174 ± 13	−12 ± 6
(II–I) ± S.E.‡	.....	−0. 4 ± 0. 6	−4 ± 9	+88 ± 12§	+91 ± 12§	+61 ± 14§	+30 ± 10‖

*Rat was sacrificed after 28 days on experimental diet.

†Standard error of the mean was estimated by the method of Mantel (*8*) in which sample range was divided by a number dependent on sample size. This number was 8.9 for the C-20 values and 9.7 for the T-24 values.

‡Standard error of the difference of the 2 means was calculated as the square root of the sum of the squares of the 2 standard errors.

§Difference significant at $P<0.001$.

‖Difference significant at $P<0.01$.

because the average carcass-weight change on the T-24 diet was greater than that on the C-20 diet (table 3).

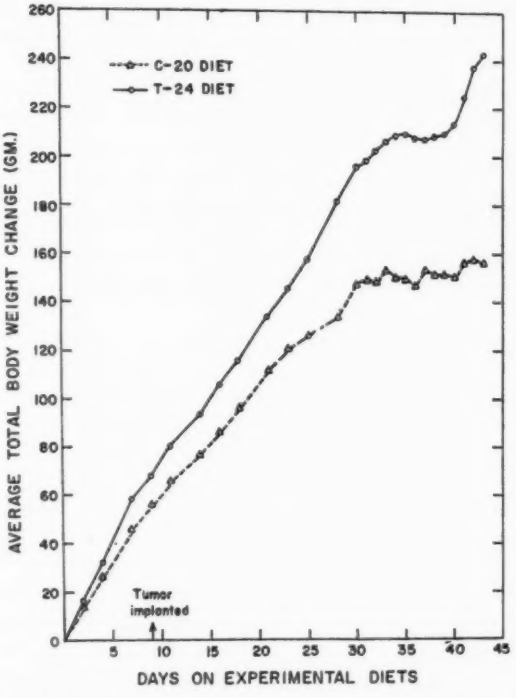
(c) *Anatomical Changes*

There were no apparent anatomical changes that could be attributed to diet. Two rats on T-24 and 3 on C-20 showed metastases in the lungs.

Generally, rats on the tumor diet have a more healthy appearance than those on the casein diet. The rat on T-24 seems more alert and better groomed, with a normal-appearing coat of fur, despite the presence of a large tumor. The coat of the C-20 animal, in contrast, often appears rough and unkempt.

(d) *Growth Effects in Preconditioned Animals*

The question has arisen whether or not the shifting of rats from chow to a synthetic diet during the course of tumor growth could in some way affect the tumor-host relationship. In an attempt to answer this, an additional experiment was carried out similar to experiment B except that the animals were placed on the synthetic diets, C-20 and T-24, 9 days prior to tumor transplant. The resulting total body-weight changes are shown in text-figure 5. The increased growth by rats on T-24 is



TEXT-FIGURE 5.—Changes in total body weight of tumor-bearing rats that were preconditioned on either the C-20 or the T-24 diet.

again evident. Food consumption by the 2 groups was essentially the same.

Discussion

Experiment A shows that the trend toward decreased food intake and body weight of tumor-bearing rats on C-25 can be reversed by the T-24 diet. This effect cannot be duplicated by diets containing lyophilized muscle or whole rat as the major source of nitrogen (*4*).

The tumors from rats fed T-24, in experiment B, weighed significantly more than those grown by rats fed the casein diet. Equally important is the smaller weight loss from the relatively normal tissues among the animals fed the tumor-containing diet. The use of lyophilized Walker carcinosarcoma 256 as a nutrient increased the net efficiency of tumor growth. This experiment was not designed to study the complete tumor-host relationship during all phases of growth of the Walker tumor. The mean measurements of the cancers grown by rats fed the different diets should be a reasonably constant function of tumor mass. The data depicted in text-figure 3 suggest that the tumors grew at equal rates for at least 15 days, but after that time the larger tumors occurred among rats on the T-24 diet.

These observations would be difficult to explain as an inherent difference in the growth vigor of the individual cancer cells. The tumor and host are interdependent. The availability of building blocks or fuels may strongly influence the growth of either host or tumor, or both. The divergence of the curves of total weight shown in text-figure 2 could represent a seriously limited supply of some essential material available to rats fed C-20 in contrast to a more adequate supply available to rats ingesting T-24. This need not be one definable substance. It could represent a series of precursors of an important compound or group of compounds. Such an effect could represent a major difference in the ratios of 2 or more substances in the cancer as compared with the relatively normal tissues.

Earlier experience has shown the ability of some cancerous rats to obtain materials from carcass tissues that are needed to supply the demands of the growing cancer (*9*). It would appear that incorporation of lyophilized Walker carcinosarcoma 256 in the diet of the rat bearing that tumor substantially reduced the contributions from normal tissues required for tumor growth.

This same general phenomenon has been studied by Haven and Bloor (*6*) who reported an increase in mass of the Walker carcinosarcoma 256 without change in food consumption when pituitary-growth hormone was administered during the course of tumor growth. They have also described an increase in total body weight of rats fed a diet that contained phospholipides extracted from the Walker carcinosarcoma (*5*).

The tumor-host relationship is dynamic and, to some extent, can be varied by controllable experimental conditions. The reproducibility of the total experience, however, is seldom absolute, as has been emphasized

previously (*4*). Reference to table 3 clearly indicates an important difference in weight relationship of rat #2654 from the rest of the group because the weight lost per gram of tumor formed was negligible.

Moreschi's general thesis (*3*) appears attractive, though none of the evidence now available indicates that any one substance is concentrated by the growing Walker carcinosarcoma nor can the idea be applied to cancers generally on the basis of one experience.

Summary

1) A semisynthetic diet (T-24), containing 24 percent lyophilized Walker carcinosarcoma 256 tissue as the source of nitrogen, was fed to Sprague-Dawley rats bearing the Walker 256 tumor.

2) In one experiment, T-24 was fed to tumor-bearing rats that had previously shown a decrease in food consumption and body weight on a 25 percent casein diet. The result was a resumption of food intake and a marked gain in weight by these animals.

3) In a second experiment, T-24 was compared, during the period of tumor growth, with a 20 percent casein diet (C-20) for effects upon total body-weight change and tumor growth. The 2 diets were approximately isocaloric and contained nearly equal amounts of nitrogen. Rats ingesting T-24 consumed the same amount of food as rats on C-20 but gained more total body weight, grew larger tumors, and showed less carcass weight loss.

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Walker Carcinosarcoma 256 Tissue as a Dietary Constituent. II. Effect Upon Growth and Nitrogen Excretion in the Normal Rat [1]

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Previous investigations (*1–4*) have demonstrated that lyophilized Walker carcinosarcoma 256 tissue, incorporated in a semisynthetic diet as a replacement for protein, exerted a stimulating effect on the body-weight change of the Walker 256 tumor-bearing rat. In the experiments reported from this laboratory (*4*), Walker 256 tumor-bearing rats were fed a diet (T-24) containing 24 percent tumor tissue and then compared with rats on a 20 percent casein control diet (C-20). The animals on T-24 grew larger tumors and showed less carcass-weight loss than the controls. In view of these effects upon both tumor and carcass, with material of unknown composition, it was of further interest to study the growth of normal rats on such a diet and to investigate the pattern of nitrogen excretion in these animals.

Materials and Methods

The effects of 2 tumor diets were studied in 2 experiments. One of these diets (T-20), which contained 20 percent lyophilized Walker 256 tumor tissue, was compared with a 20 percent casein diet (C-20). In the second test diet (T-24), the tumor content was increased to 24 percent so that the 2 diets might contain equivalent amounts of nitrogen while remaining isocaloric.

The composition of C-20 and T-24 has been given previously (*4*). The composition of T-20 was the same as that of C-20 except for the substitution of tumor tissue for casein as noted. The nitrogen content and caloric value of the diets are given in table 1.

Weanling male Sprague-Dawley rats were allowed to consume Purina chow diet until they reached a weight of about 80 gm. The animals were then divided into 2 groups and were fed either the tumor diet (T-20 or T-24) or the control diet (C-20).

All the animals were individually housed. Food intake and animal weights were recorded daily. Animals eating *ad libitum* were used in growth studies. Pair-fed animals were used in growth and nitrogen-excretion

[1] Received for publication July 9, 1957.

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TABLE 1.—*Total nitrogen content and heat of combustion of diets*

Diet	Total nitrogen (*mg./gm. of diet*)	Heat of combustion (*cal./gm. of diet*)
C-20	29. 3	5. 32
T-24		
(0–18 days)	29. 4	5. 17
(19–23 days)	30. 3	5. 36
T-20	25. 4	—

studies as follows: In the test of the T-20 diet, 3 rats on T-20 were pair fed to 3 rats on C-20; in the test of the T-24 diet, 4 rats on T-24 were pair fed to 2 rats on C-20.

These pair-fed rats and their controls were kept in metabolic cages from which both urine and feces were collected quantitatively. Total urinary nitrogen was determined by the micro-Kjeldahl method, with mercuric oxide and selenium (Hengar granules) as catalysts, urea as the dixanthhydrol derivative, allantoin by the method of Young and Conway (*5*), and ammonia by absorption on permutit.

Results and Discussion

Weight Gain by Rats Fed Ad Libitum

The average weights and food intake of animals in the 2 experiments, over a 15-day period, are summarized in table 2. It can be seen that animals on either the T-20 or T-24 diet consumed food and gained weight like their respective controls. On either a weight basis or a total nitrogen basis, it would appear that the inclusion of lyophilized Walker tumor in the diet is as effective as casein as a source of protein for the growth of the young rat.

Weight Gain and Nitrogen-Excretion Pattern by Pair-Fed Rats

Since the 2 tumor diets, T-20 and T-24, produced essentially similar effects upon the weight gain and nitrogen-excretion pattern of animals pair fed to casein controls, only the results from the T-24 experiment are presented in detail here. The average weight gain of 4 rats ingesting the T-24 diet and of 2 rats ingesting the C-20 diet, over a period of 23 days, are shown in text-figure 1. The 2 growth curves coincide, and the over-all increase in weight is the same for the 2 groups.

The total nitrogen intake for these 2 groups over the 23-day period was an average of 6.39 gm. per rat for the T-24 rats and 6.57 gm. per rat for the C-20 controls due to the slight difference in nitrogen content of the 2 diets (table 1).

Text-figure 2 shows the daily total urinary-nitrogen excretion of the 2 groups. Although there are differences between points on the curves, these are not considered significant and the over-all shapes of the 2

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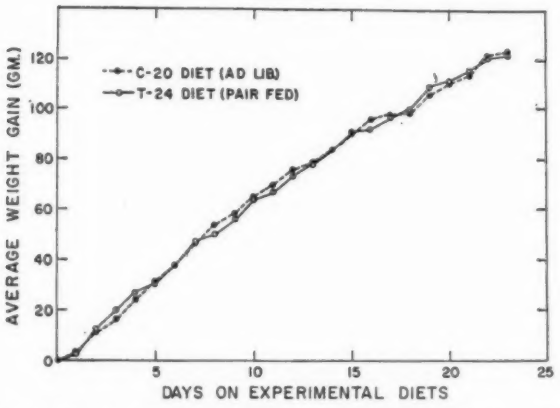
TABLE 2.—*Body weight and food consumption of rats ingesting* ad libitum *either of 2 tumor diets (T-20 or T-24) or a casein diet (C-20) for a 15-day period**

Expt. no.	Diet	No. of animals	Initial weight (*gm.*)	Final weight (*gm.*)	Average weight gain (*gm./day*)	Average food consumption (*gm./day*)	Gm. gained / Gm. food eaten
1	C-20	15	80. 1 ± 0. 3	180. 9 ± 3. 1	6. 7 ± 0. 2	11. 8 ± 0. 2	0. 57 ± 0. 01
	T-20	15	80. 9 ± 0. 4	185. 7 ± 4. 0	7. 0 ± 0. 3	12. 2 ± 0. 3	0. 57 ± 0. 01
2	C-20	10	79. 7 ± 0. 5	174. 2 ± 2. 7	6. 3 ± 0. 2	10. 5 ± 0. 2	0. 60 ± 0. 01
	T-24	10	80. 2 ± 0. 4	181. 0 ± 4. 2	6. 7 ± 0. 3	11. 0 ± 0. 3	0. 61 ± 0. 00

*Mean of animals ± Standard Error.



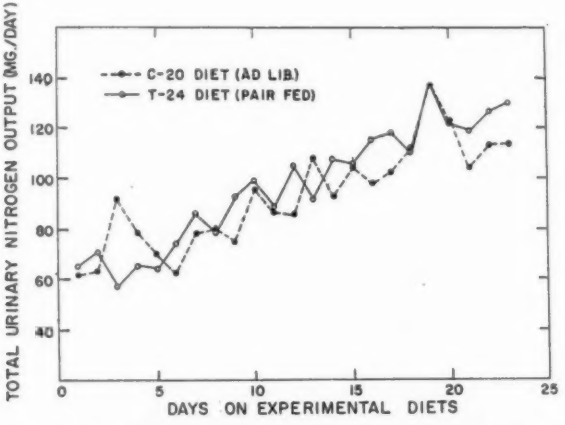
TEXT-FIGURE 1.—Weight gain of rats ingesting either the C-20 diet (*ad libitum*) or the T-24 diet (pair-fed).

curves are the same. The total urinary excretion over the 23-day period was an average of 2.23 gm. nitrogen per rat for the T-24 rats and 2.14 gm. per rat for the C-20 controls.

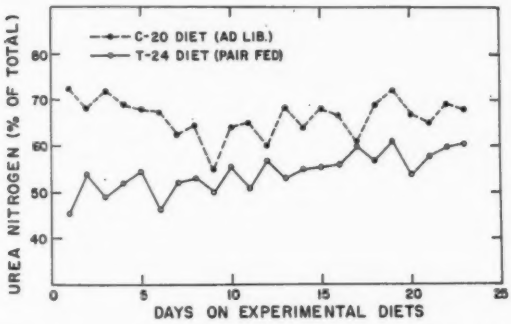
Examination of the urea-excretion data (text-fig. 3) reveals a notable difference in the output by the 2 groups of rats. Animals ingesting the T-24 diet excreted far less of their total urinary nitrogen as urea.

Ammonia excretion in the 2 groups was approximately the same (7–10%). The remainder of the urinary-nitrogen excretion represents chiefly allantoin and creatinine.

Text-figure 4 shows the allantoin nitrogen excretion of the 2 groups of rats. Animals on the T-24 diet consistently excreted more total nitrogen as allantoin than animals on C-20.



TEXT-FIGURE 2.—Total urinary-nitrogen output of rats ingesting either the C-20 diet (*ad libitum*) or the T-24 diet (pair-fed).

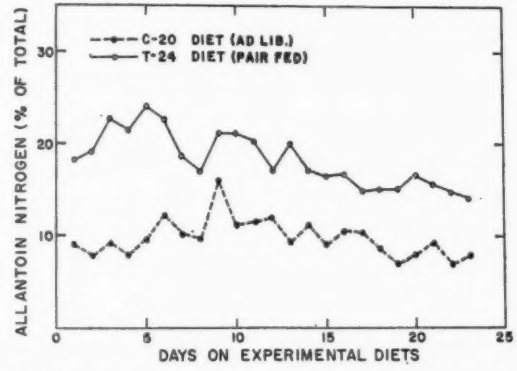


TEXT-FIGURE 3.—Urinary urea nitrogen of rats ingesting either the C-20 diet (*ad libitum*) or the T-24 diet (pair-fed).

Associated with the high excretion of allantoin from rats on the T-24 diet was a high urinary phosphorus : nitrogen (P:N) ratio as compared with a relatively low ratio from animals ingesting the C-20 diet. The urinary P:N ratios, averaged over the 23-day period, were 0.119 and 0.018 for rats ingesting the T-24 and C-20 diets, respectively. The P:N ratio in the C-20 diet itself was found to be 0.089, whereas the ratio in the T-24 diet was 0.170.

It may be noted here that in the experiment in which 3 rats on T-20 were pair fed to 3 rats on C-20, the former excreted only 30 to 40 percent of their total urinary nitrogen as urea, in contrast to 60 to 70 percent excreted by the controls. Corresponding figures for urinary allantoin nitrogen were approximately 35 and 10 percent. Urinary P:N ratio was higher for T-20 rats.

The protein and nucleic acid content of the Walker 256 tumor tissue are of interest in the light of these results. Treatment of lyophilized Walker 256 tumor with hot 1 *M* NaCl yielded a protein fraction that was 51 percent of the starting material and contained 14.1 percent nitrogen.



TEXT-FIGURE 4.—Urinary allantoin nitrogen of rats ingesting either the C-20 diet (*ad libitum*) or the T-24 diet (pair-fed).

Schneider and Klug (*6*) found that every 100 gm. of Walker 256 tumor (wet weight) contained about 1.3 gm. of nucleic acid. On a dry-weight basis this is calculated to be about 6 to 7 percent. Assuming that this nucleic acid contains 16 to 17 percent nitrogen, one can then calculate that, of the 30 mg. total nitrogen available per gm. of T-24 diet, about 20 mg. can be accounted for as protein and nucleic acid. In contrast, 30 mg. of protein nitrogen (from casein) is provided per gm. of C-20 diet. In spite of this difference, the T-24 diet is as effective as the C-20 diet in promoting the growth (body-weight change) of young normal rats.

The analysis for protein content of tumor is altogether in line with the results of the excretion studies. Lowered urea nitrogen excretion by rats on either of the 2 tumor diets relative to the casein controls reflects the lower protein content of tumor and suggests that the animals are retaining a greater proportion of the dietary protein nitrogen.

Conversely, both the high allantoin nitrogen and P:N ratio of the urine from rats on the tumor diets, in comparison with the casein controls, indicate a higher metabolism of nucleic acids or nucleic acid derivatives, presumably metabolized directly from the diet. From the results for the nucleic acid content of the tumor tissue (*6*) and the total nitrogen intake per rat on the T-24 diet, one can make a rough calculation of the total allantoin nitrogen, which could come from direct metabolism of nucleic acid in the tumor diet. This amounts to about 300 mg. of nitrogen and is more than enough to account for the excess in allantoin nitrogen (about 180 mg.) actually excreted by the T-24 rat over that excreted by the C-20 control during the course of the experiment.

While the excretion studies serve to point up the difference in protein content of tumor tissue and casein, they do not furnish an explanation for the equal growth obtained on the 2 diets despite this difference. Whether or not the protein of tumor tissue is more "complete" than casein is unknown at the present time. Tumor tissue may contain constituents that are capable of leading to nucleic acid synthesis, which in turn may spare protein, while animals on the casein diet must use the protein for both protein and nucleic acid synthesis. Whether or not the protein isolated from tumor tissue will support growth as well as casein is being studied.

Meanwhile, it is still possible that other constituents of the tumor may be responsible for the observed growth effects. Thus, constituents of the fat fraction or of the nucleic acid fraction, hormones, inorganic elements, or some combination of these substances may provide the answer. Haven and Bloor (*2*) have shown that an active principle, which restores appetite and increases total body weight of the tumor-bearing rat, resides in the phospholipide fraction of the Walker tumor.

Summary

1) Two semisynthetic diets, containing either 20 percent or 24 percent lyophilized Walker carcinosarcoma 256 tissue instead of casein as a source of nitrogen, were tested in young Sprague-Dawley rats for their effects

on growth and excretion products. One of these diets (T-20) contained an amount of tumor tissue equal in weight to the casein (20%) in the casein control diet (C-20). The other diet (T-24) contained an amount of total nitrogen equal to that of the casein diet (C-20) and was isocaloric with it. Lyophilized Walker 256 tumor contained about 51 percent protein.

2) Growth of rats on either T-20 or T-24 was equal to that of their C-20 controls both when food consumption was *ad libitum* and when animals on the tumor diets were pair fed to the controls.

3) When rats ingesting either of the 2 tumor diets were pair fed to rats ingesting the casein diet, the following differences were observed in the urinary excretions:

a. Urea nitrogen excretion of animals ingesting the tumor diets was consistently less than that of their C-20 controls.
b. Allantoin nitrogen excretion of animals ingesting the tumor diets was consistently greater than that of their controls.
c. Ammonia nitrogen excretion of animals ingesting the tumor diets was approximately the same as that of their C-20 controls.
d. The urinary phosphorus:nitrogen ratio was considerably higher for animals on the tumor diets than for those on the C-20 diet.

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Cocarcinogenic Activity of Cholesterol Oxidation Products and Sesame Oil [1, 2]

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The observation (*1*) reported from this laboratory, that a crude progesterone preparation (*2*) made by permanganate oxidation of cholesterol dibromide and debromination produced a 32 percent incidence of local malignant tumors in Marsh-Buffalo mice, prompted an extended investigation of the relation of cholesterol oxidation products to carcinogenesis. The purpose of this communication is to show that certain oxidation products of cholesterol give rise to a high incidence of fibrosarcoma[5] when injected subcutaneously into Marsh-Buffalo mice with sesame oil as the vehicle. Data on the effect of injection of these steroids as aqueous colloids or suspensions are included, but the influence of these substances when injected in other media will be taken up at a subsequent date.

The ease with which cholesterol is oxidized in aqueous colloidal solution by air simulates a condition that could conceivably be duplicated *in vivo*. For this reason, the known oxidation products or mixtures of them have been tested for carcinogenic activity. Eight known compounds have been isolated (*7*, *8*) and 5-cholesten-3-one has been postulated. The 8 compounds are: 7-oxocholesterol; 7α-hydroxycholesterol; 7β-hydroxycholesterol; cholestane-3β,5α,6β-triol; 3,5-cholestadien-7-one; 6-cholestene-3β,5α-diol; 4-cholesten-3-one; and dihydrocholesterol. 7-Oxocholesterol was prepared in this laboratory, 4-cholesten-3-one was obtained from Schering Corporation, and cholestane-3β,5α,6β-triol and 5-cholesten-3-one were obtained from Louis F. Fieser. The other compounds were tested as a mixture in either the keto or nonketo fractions of the oxidation-reaction product of cholesterol prepared in this laboratory according to the directions of Bergström and Wintersteiner (*7*) and separated according to the standard Girard procedure.

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[1] Received for publication June 14, 1957.

[2] This work was supported in part by research grant C-1586 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

[3] With the technical assistance of Eugene W. Sheller, Guillermo Lopez, and Robert A. Fraundorf. We are indebted to Dr. J. J. Rupp for the histologic reports, to Dr. E. L. Benjamin and Dr. D. R. Dickson for histologic advice, and to Dr. Louis F. Fieser for most of the steroids tested. This study would not have been possible without Dr. Fieser's help and interest.

[4] We are indebted to the Schering Corporation for the 4-cholesten-3-one and for 4-androstene-3,17-dione, and to Dr. C. D. Larsen for the highly purified sample of cholesterol.

[5] Preliminary reports have been published (*3-6*).

A number of compounds tested in this study were obtained from Louis F. Fieser and were suggested by specific hypotheses of Fieser *et al.*: 7-cholesten-3β-ol (lathosterol), 7,9(11)-cholestadien-3β-ol acetate[6] and 7,8,9,11-diepoxy-22-isoallospirostan-3β-ol acetate,[7] more or less related compounds containing or capable of forming mono or diepoxide groups (*9*); cholesteryl acetoacetate and cholesteryl isoheptylate, unusual esters of cholesterol (*10*).

Finally a number of compounds were tested as possible by-products of the original cholesterol dibromide oxidation, or of any cholesterol oxidation. These included: 4-cholestene-3,6-dione; 1,4-cholestadien-3-one; 6β-hydroxy-4-cholesten-3-one; 5α,6α-epoxy-3β-cholestanol;[8] 6β-hydroperoxy-4-cholesten-3-one; cis 5-cholestene-3,4-diol; and 4-androstene-3,17-dione.

Experiments

Marsh-Buffalo male and female mice, either castrated or intact, were employed as the test animals. Castration was performed before 30 days of age. Groups of approximately 30 mice were used per compound. When injections were made in sesame oil, the first injection was made at 3 months of age, and 1 or 2 subsequent injections were made at 2-month intervals. The total amount of steroid administered in sesame oil per mouse was 15 to 20 mg. The sesame-oil solution was heated on the water bath for 10 minutes and consisted of 7 ml. sesame oil, 350 mg. steroid, and 1 ml. ethanol. In some instances the resulting solution is supersaturated and therefore injection was made before crystallization took place. The injections in all cases were dorsal and were given subcutaneously, as far as possible, from the mammary-gland areas. For the series of sesame-oil controls *see* Results. A number of steroids were given in colloidal solution or suspension. One procedure of preparation was to add an ethanol solution or part solution of the steroid to water so that the final ethanol concentration was 2 percent. Another method was to grind 200 mg. steroid, 16 ml. water, and 160 mg. bovine albumin. Steroids given in aqueous suspension were administered 5 to 15 mg. per mouse.

The experiments were terminated at 18 months of age. The recording and presentation of tumor data has been previously described (*1*). It did not seem feasible in this report to present the data on increase in cancer incidence per month, because only the final incidence is pertinent for interpretation.

Three batches of sesame oil, obtained from 3 sources (Brunswig Drug Company, Pacific Coast Drug and Chemical Corp., and Magnus, Mabee & Reynard, Inc.), were tested as controls for sesame oil alone at various times throughout the period of experimentation and tests for carcinogenic activity of cholesterol derivatives were made simultaneously. The dregs of a bottle of sesame oil which had been in the laboratory for 15 years were also tested. An experiment with sesame oil that had air bubbled through

[6] Formerly designated $\Delta^{7,9(11)}$-cholestadienyl acetate.
[7] Formerly designated 7,8,9,11-dioxide-22-isoallospirostan-3β-ol acetate.
[8] Formerly designated cholestrol-α-oxide.

it for 44 hours at room temperature is included. This procedure formed a product that gave a positive test for peroxide with KI (1.0 ml. 0.01 *N* thiosulfate per ml. oil). The original oil gave a negative test.

Sections of the tumors were submitted to the histologists in code to eliminate bias.

Results

Sesame-oil control.—Data were obtained on 355 mice carried to the age of 17 to 18 months on the effect of subcutaneous dosage of 3 different batches of sesame oil. These include castrated and intact male and female mice. The incidence of fibrosarcoma was 1.4 percent. There is no indication from the data that sex or castration affected the response to sesame oil, nor is there any concrete evidence that the incidence for sesame oil is greater than that of untreated mice. In 40 undosed males carried to 18 months of age, there was no fibrosarcoma. In 126 castrated males carried to 17 to 18 months, 2 developed fibrosarcoma. When the dregs of old sesame oil or sesame oil, which had been aerated to produce peroxide, were tested, the incidence of fibrosarcomas was 9 percent for 63 mice. These series are sufficiently large to warrant the definite conclusion that sesame oil itself on subcutaneous injection is not carcinogenic to Marsh-Buffalo mice, but that the aerated sesame oil is mildly carcinogenic (table 1).

If the incidence of fibrosarcoma, 1.2 ± 0.7 percent in 248 mice carried to 18 months of age, is taken as the sesame-oil control incidence for the colony, an incidence below 9 percent in a group of 32 mice would clearly be without significance. Figures between 9 and 15 percent would have doubtful significance. Figures above 25 percent would be highly significant. The data have been divided into 2 groups in tables 2 and 3 according to significance of results.

Chemical structure and fibrosarcoma production.—Tables 2 and 3 give completed long-term experiments on the production of fibrosarcomas by the various steroids with sesame oil as the vehicle injected subcutaneously into Marsh-Buffalo mice. Note the highly significant results for 4 com-

TABLE 1.—*Tumor incidence of Marsh-Buffalo mice injected with oil vehicles*

Vehicle	No. of mice and sex*	Accumulative incidence to age 18 months (*percent*)			
		Adeno-carci-noma	Lymphoid tumors	Fibro-sarcoma	Death other causes
Sesame oil	79 M intact	0	5	0	33
Sesame oil	31 M castrated	0	6	0	12
Sesame oil	66 F castrated	13	9	1. 5	15
Sesame oil	72 F intact	64	7	3†	7
Sesame oil, 15 yrs. old	31 M castrated	0	3	12	9
Sesame oil, aerated	32 F castrated	0	3	6	9
Lard, peroxide #8	32 M intact	0	3	0	25

*M indicates males, F indicates females.

†Two percent fibrosarcoma in an additional 107 females taken to age 17 months.

TABLE 2.—*Tumor incidence of Marsh-Buffalo mice injected with sesame oil and steroids (negative results)*

Steroid	No. of mice and sex*	Accumulative incidence to age of 18 months (*percent*)			
		Adeno-carci-noma	Lymphoid tumors	Fibro-sarcoma	Death other causes
7,9(11)-cholestadien-3β-ol acetate†	32 M	0	12	0	12
7,8,9,11-diepoxy-22-iso-allospirostan-3β-ol ace-tate	33 M	0	0	0	12
Purified cholesterol	20 M castrated	0	45	0	15
Purified cholesterol	35 F	57	15	0	6
4-Cholesten-3-one	34 F	68	12	3	0
4-Cholestene-3,6-dione	33 F castrated	15	3	3	24
7-Cholesten-3β-ol (lathos-terol)	48 F castrated	8	8	6	14
Pure progesterone	35 F castrated	0	15	0	3
4-Cholesten-3-one	35 F castrated	0	32	0	12
4-Androstene-3,17-dione	33 F castrated	33	0	3	15

*M indicates males; F indicates females.
†Similar results when lard with peroxide #8 was used as vehicle.

pounds or fractions: "oxy cholesterol"; 4-cholestene-3,6-dione; 5α,6α-epoxy-3β-cholestanol; and 6β-hydroperoxy-4-cholesten-3-one. Note also that the highly significant results for 5α,6α-epoxy-3β-cholestanol were confirmed in the experiment in which a sesame-oil concentrate was added to a cottonseed-oil vehicle (*6*).

Steroids as aqueous colloids or suspensions.—The following compounds were given as aqueous colloids in experiments, with 30 or more mice per experiment, carried to the age of 18 months with a satisfactory survival rate and with negative results (0 or 3%) as far as production of fibrosarcomas was concerned: purified cholesterol (64 mice); 7,9(11)-cholestadien-3β-ol acetate (34 mice); 7,8,9,11-diepoxy-22-isoallospirostan-3β-ol acetate (35 mice); 5-cholesten-3-one; and cholestane-3β,5α,6β-triol. Negative results (3%) were also obtained for 5α,6α-epoxy-3β-cholestanol and 6β-hydroperoxy-4-cholesten-3-one in experiments carried to 15 months of age, after which there was a high incidence of deaths due to an epidemic of pneumonia. At the age of 15 months 5α,6α-epoxy-3β-cholestanol produced a 39 percent incidence of fibrosarcoma when administered in sesame oil, a 33 percent incidence when administered in cottonseed oil plus sesamin mother liquor, and an 18 percent incidence in cottonseed oil. 6β-Hydroperoxy-4-cholesten-3-one produced a 56 percent incidence when administered in sesame oil. While it is unfortunate that the experiments

TABLE 3.—*Tumor incidence of Marsh-Buffalo mice injected with sesame oil and steroids (positive results)*

Steroid	No. of mice and sex*	Accumulative incidence to age of 18 months (*percent*)			
		Adeno-carci-noma	Lymphoid tumors	Fibro-sarcoma	Death other causes
Eastman cholesterol	64 M	0	9	13	17
Nonketo fraction, B&W, "oxy cholesterol"	31 M	0	10	23	20
B&W "oxy cholesterol"	34 M	0	15	9	23
4-Androstene-3,17-dione	64 M	0	5	13	25
6β-Hydroxy-4-cholesten-3-one, epicholesterol 1:1	32 M	0	0	19	25
4-Cholestene-3,6-dione	32 M	0	0	34	28
7-Oxocholesterol	32 M	0	3	9	3
6β-Hydroperoxy-4-cholesten-3-one	32 M castrated	0	3	66	15
7-Cholesten-3β-ol (lathosterol)	20 M castrated	0	11	15	0
Nonketo fraction, B&W, "oxy cholesterol"	33 F castrated	18	3	9	21
5α,6α-Epoxy-3β-cholestanol	33 F castrated	9	0	48	9
6β-Hydroxy-4-cholesten-3-one, epicholesterol	33 F castrated	18	0	15	15
Cholesterol isoheptylate	33 F castrated	30	6	9	27

*M indicates male; F indicates female.

involving administration of the aqueous colloids of these compounds are valid only to the age of 15 months, the marked difference with the results for the oily vehicles at the age of 15 months is nevertheless conclusive.

Fibrosarcoma transplants.—No attempt has been made to transplant every fibrosarcoma that has developed in our experimental work (over 200). However, since this is one of the criteria of malignancy, transplants from 12 different tumors were made subcutaneously in each of 2 Marsh-Buffalo mice. The 12 tumors were selected in the order of their appearance. One tumor did not take. The growth of the transplants from 10 tumors was extremely rapid after a variable latent period. Histologically, the transplant resembled the respective parent tumor. Thirty days after transplantation, 10 mg. transplants grew to a mean diameter between 2 and 3 cm.

Discussion

Fibrosarcomas.—Fibrosarcoma occurs spontaneously in the mouse, arising in the subcutaneous region from the fibrous connective-tissue cells

(*11*). Abundant mitotic figures, pleomorphic forms, invasion of striated muscle, *etc.*, areas of necrosis, and extremely rapid growth are characteristic. Considerable variation in histologic appearance is observed.

Steiner *et al.* (*12*) found that sesame oil heated to 350° C. was carcinogenic (3 of 9 mice), while unheated sesame oil was not. The tumors he described, as spindle-cell or spindle-cell and mixed-cell sarcoma, meet the qualifications of the fibrosarcoma. They infiltrated both the overlying skin and underlying muscle and were adjacent to residual sesame oil. They did not metastasize.

Dunn *et al.* (*13*) studied the spontaneous occurrence of fibrosarcoma in a series of 4,049 untreated female mice of various strains. The sarcomas occurred in mice of very advanced age. In one group a sarcoma appeared at 17 months, but in all other groups the average age was over 20 months; in a few it was over 30 months. These observations are in line with those previously reported from this laboratory (*1*), in which a 10 percent incidence in 30 mice was found at the age of 20 months or over. In the present studies, the experiments were terminated before the age at which spontaneous fibrosarcoma development would be a complicating factor in establishing the carcinogenic effect of injected substances.

The fibrosarcomas obtained in these studies coincide macroscopically and histologically with the references given (*see* figs. 1 to 6). They arose at the injection site, frequently lay adjacent to or even surrounded the oleoma formed around the injected oil. However, in some cases the oil had disappeared. On blunt dissection ("skinning") they tended more often to adhere to the skin rather than to the muscle layer. Formation of the fibrosarcoma from the cells of the fibrous-tissue capsule of the oleoma was definitely indicated in some cases.

The question of whether or not pure cholesterol is carcinogenic under our experimental conditions must be considered. Hieger (*14–16*) observed that subcutaneous injection of cholesterol in lard in several hundred mice produced a 4 to 6 percent incidence of malignant tumors at the site of injection. The appearance of these tumors was at a late date. In our series of 64 male mice carried to 18 months and given Eastman cholesterol in sesame oil, the incidence of fibrosarcoma was 13 percent. Since the incidence for the control sesame oil in 79 male mice was 0 percent the result is significant, and would also be significant if the incidence of 1.2 percent were taken for the colony as a whole. This Eastman cholesterol

TABLE 4.—*Correlation of oleoma and fibrosarcoma*

Compound	No. of mice	Oleoma (*percent*)	Fibrosarcoma with oleoma (*percent*)	Fibrosarcoma without oleoma (*percent*)
6β-Hydroperoxy-4-cholesten-3-one	32	28	20	45
4-Cholestene-3,6-dione	32	56	22	13
4-Cholestene-3,6-dione	33	49	3	0
5α,6α-Epoxy-3β-cholestanol	32	18	12	37

had a m. p. of 146.5 to 147.5° C. and contained 0.5 percent 7-cholesten-3β-ol (lathosterol). The results with a purified cholesterol, m. p. 149° C., that contained less than 0.2 percent 7-cholesten-3β-ol follow: When injected subcutaneously in 64 mice (30 males, 13 castrated females, and 21 castrated males) as an aqueous colloid, the results were completely negative at 18 months of age. When injected in sesame oil into 70 mice (35 females, 15 castrated females, and 20 castrated males), the incidence for purified cholesterol was 3 percent, which would not be significant. When 7-cholesten-3β-ol in sesame oil was injected (48 castrated females and 20 castrated males) the 9 percent incidence of fibrosarcoma would probably be significant for the group, but not for individual experiments.

The 3 compounds which proved to have the greatest carcinogenic effect, 4-cholesten-3,6-dione, 5α,6α-epoxy-3β-cholestanol, and 6β-hydroperoxy-4-cholesten-3-one, all share the property of having an oxygen linkage at carbon 6. 6β-Hydroxy-4-cholesten-3-one was mildly carcinogenic. In 1 experiment with 4-cholestene-3,6-dione the results were negative. However, in 2 experiments with cottonseed oil the carcinogenic effect of 5α-6α-epoxy-3β-cholestanol was confirmed. Since the results with an aqueous vehicle were negative and since oleomas formed prior to the development of the fibrosarcoma in most experiments and frequently were adjacent to or surrounded by the fibrosarcoma, we once felt that the development of oleoma with its fibrous-tissue capsule, was a requirement for the subsequent development of a fibrosarcoma. However, this concept proved erroneous (table 4), for in the case of 6β-hydroperoxy-4-cholesten-3-one, which produced a 66 percent incidence of fibrosarcoma, oleoma formation did not take place for two thirds of the tumors. Similar results were obtained for 5α,6α-epoxy-3β-cholestanol. In this connection, it is interesting that Orr (*17*) felt that the development of sarcoma following implanted paraffin pellets containing carcinogenic hydrocarbons was related to a deficient or frustrated foreign-body response occasioned by the presence of the carcinogen.

Between pure cholesterol, which has not proved to be carcinogenic, and 6β-hydroperoxy-4-cholesten-3-one, which was highly carcinogenic, lies a group of compounds that produced an intermediate effect when administered in sesame oil. Among these are the oxidation products of cholesterol produced by bubbling air through a colloidal solution with soap as the dispersing agent, also 4-androstene-3,17-dione and deoxycorticosterone reported by another laboratory (*18*).

Since 4-androstene-3,17-dione and deoxycorticosterone are normal body constituents, they could hardly be regarded as carcinogenic *per se* and the conclusion to be drawn from the data at hand is that sesame oil acts as a cocarcinogen, either by containing a constituent which reacts with the cholesterol oxidation products or by producing an environment (the oily drops, the subsequent oleoma, delayed resorption, *etc.*) in which chemical transformation with endogenous constituents reaching the oily medium takes place. The reasons for regarding sesame oil as a cocarcinogen may be reiterated: 1) It is not carcinogenic. 2) The heated or

oxidized oil is mildly carcinogenic. 3) Certain oxidation products of cholesterol are highly carcinogenic when administered in sesame oil, noncarcinogenic when administered in aqueous suspensions. 4) Certain steroids which are normal body constituents are carcinogenic when administered in sesame oil. Comparative studies of the effect of different vehicles on the carcinogenic action of the carcinogenic hydrocarbons place sesame oil high on the list of those substances producing an enhanced effect and sesame oil has been dubbed a cocarcinogenic agent (*19*, *20*). That sesame oil is able to produce the carcinogenic effect in combination with normal biologic constituents should stimulate interest in a study of the cocarcinogenic properties of sesame-oil fractions and constituents.

Summary

1) Sesame oil or purified cholesterol dissolved in sesame oil are not carcinogenic following subcutaneous injection into Marsh-Buffalo mice.

2) A number of oxidation products of cholesterol, when administered with sesame oil as the vehicle, are carcinogenic. The most potent found to date are 4-cholestene-3,6-dione, 5α,6α-epoxy-3β-cholestanol, and 6β-hydroperoxy-4-cholesten-3-one. The latter compounds have in common an oxygen linkage at carbon 6.

3) Oxidation products of cholesterol that were carcinogenic when administered in a sesame-oil vehicle were noncarcinogenic when administered as aqueous colloids.

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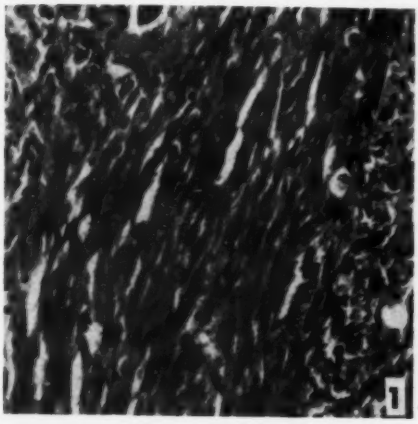
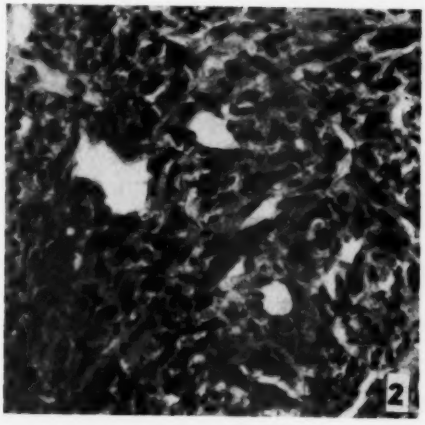
PLATE 84

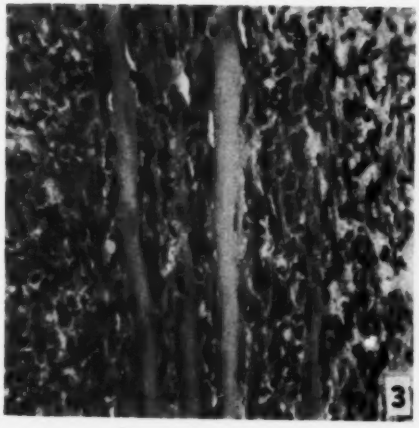
FIGURES 1, 2, 4, and 5.—Sections of fibrosarcomas produced locally by injecting 6β-hydroperoxy-4-cholesten-3-one in sesame oil to Marsh-Buffalo mice. Hematoxylin and eosin. × 400

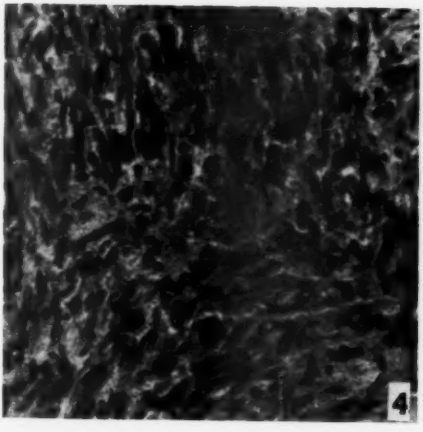
FIGURE 3.—Section of fibrosarcoma produced locally by injection of 5α,6α-epoxy-3β-cholestanol in cottonseed oil to Marsh-Buffalo mouse. Hematoxylin and eosin. × 200

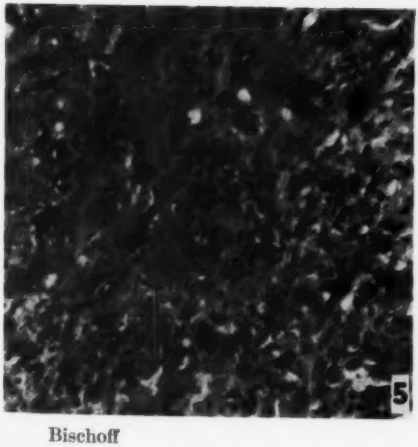
FIGURE 6.—Transplant in a Marsh-Buffalo mouse of a fibrosarcoma produced locally by injection of 5α,6α-epoxy-3β-cholestanol and sesamin concentrate in cottonseed oil. Hematoxylin and eosin. × 400

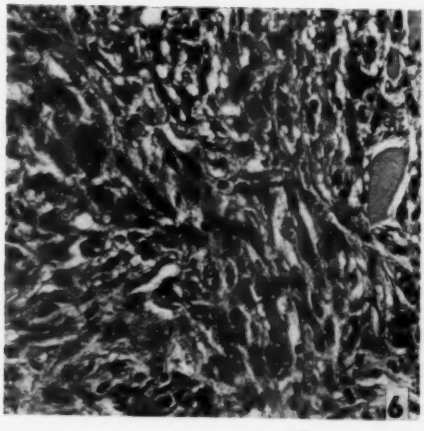












Bischoff

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ANNOUNCEMENT

Seventh International Cancer Congress, London, 1958

Those planning to attend the Seventh International Cancer Congress, which will take place at the Royal Festival Hall, London, from July 6 to July 12, 1958, are reminded that enrollment forms must be received at the Congress Office (45 Lincoln's Inn Fields, London, W. C. 2) by *JANUARY 1, 1958*, if a late fee is not to be incurred. Registration forms may be obtained from the Secretary-General at that address.

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At the request of the Senate Appropriations Committee, the National Institutes of Health has initiated, developed, and supported the translation and publication of selected Russian literature in the biological and medical sciences. The purpose of this Program is to inform American scientists concerning recent Soviet advances in the biomedical sciences. The Program includes:

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ERRATUM

Volume 19, Number 1, July 1957
Journal of the National Cancer Institute

In legend of the paper entitled "Regression of HeLa tumors in cortisone-treated irradiated rats following infection with Coxsackie B3 virus. A histological and cytochemical study." by Love, Suskind, and Huebner:

PAGE 72, PLATE 11, FIRST LINE of legend for Figure 21 *for* "Higher magnification of the tumor shown in figure 20." *read* "Higher magnification of the tumor shown in figure 19."

U. S. GOVERNMENT PRINTING OFFICE: 1957

The Journal of the National Cancer Institute is published monthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Twelve issues are published each year in two volumes, beginning with number 1 in July and January. The Journal publishes original observations and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

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Use of funds for printing this publication approved by the Director of the Bureau of the Budget, May 14, 1956

UNITED STATES GOVERNMENT PRINTING OFFICE, WASHINGTON : 1957

For sale by the Superintendent of Documents, U. S. Government Printing Office
Washington 25, D. C. – Price per copy $2.00.

Subscription price for two volumes per year in the United States, Canada, and Mexico, $20.00; foreign, $25.00.

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